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(54) Title: RECOMBINANT RSV WITH SILENT MUTATIONS, VACCINES, AND METHODS RELATED THERETO

AA	Codon	Human Brain Genes	A2	2-20	Line19	3-2	3-4	12-21
	GGT							
Ala	GCC							
	GCA							
	GCG							
Cys	TGT							
	TGC							
Asp	GAT							
	GAC							
Glu	GAA							
	GAG							
Phe	TTC							
	TTG							
Gly	GGT							
	GGC							
	GGG							
His	CAT							
	CAC							
Ile	ATT							
	ATC							
Lys	AAA							
	AAG							
	TAA							
Leu	TTC							
	CTT							
	CTG							
	CTC							
Asn	AAU							
	AAC							

AA	Codon	Human Brain Genes	A2	2-20	Line19	3-2	3-4	12-21
	CCT							
Pro	CCC							
	CCA							
	CCG							
Gln	CAA							
	CAG							
	CGT							
	CGC							
Arg	CGA							
	CGG							
	AGA							
	AGG							
	TCT							
	TCC							
Ser	TCA							
	TCC							
	AGT							
	AGC							
	ACT							
	ACC							
Thr	ACA							
	ACG							
	GTG							
	GTC							
	GTA							
	GTG							
	TAT							
Tyr	TAC							

FIG. 1

(57) **Abstract:** In certain embodiments, the disclosure relates to the polynucleotide sequences of respiratory syncytial virus (RSV). In certain embodiments, the disclosure relates to isolated or recombinant nucleic acids and polypeptides comprising desirable nucleic acid sequences and mutations disclosed herein. In certain embodiments, isolated or recombinant RSV comprising the nucleic acids and polypeptides disclosed herein (e.g., attenuated recombinant RSV) are also provided, as are immunogenic compositions including such nucleic acids, polypeptides, and RSV genomes that are suitable for use as vaccines. Attenuated or killed RSV containing these nucleic acids and mutation in the form of copied nucleic acids (e.g., cDNAs) are also contemplated.



- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

RECOMBINANT RSV WITH SILENT MUTATIONS, VACCINES, AND
METHODS RELATED THERETO

CROSS REFERENCE TO RELATED APPLICATIONS

5 This Application claims priority to U.S. Provisional Application Number 61/781,228 filed March 14, 2013 and U.S. Provisional Application Number 61/890,500 filed October 14, 2013, both incorporated by reference in their entirety.

BACKGROUND

10 Respiratory syncytial virus (RSV) leads to lower respiratory tract infections. Immunocompromised patients, premature infants, and children are particularly at risk to severe disease. RSV is the leading cause of viral death in infants. RSV treatments are focused on prevention from infection and improving respiration. Palivizumab is a humanized monoclonal antibody that can be given prophylactically. Palivizumab is not 15 effective after RSV infection, and protection ends shortly after treatment stops. Vaccines are not currently available for RSV. Attenuated RSV vaccines candidates have failed because of suboptimal immunogenicity in infants and suboptimal stability that leads to genetic reversion towards undesirable wild-type sequences. See Teng, *Infectious Disorders – Drug Targets*, 2012, 12(2):129-3. Thus, there is a need to find an attenuated RSV vaccine 20 that is appropriately immunogenic, sufficiently stable, and safe for use in infants.

Due to the redundancy of the genetic code, individual amino acids are encoded by multiple sequences of codons, sometimes referred to as synonymous codons. In different species, synonymous codons are used more or less frequently, sometimes referred to as codon bias. Genetic engineering of under-represented synonymous codons into the coding 25 sequence of a gene has been shown to result in decreased rates of protein translation without a change in the amino acid sequence of the protein. Mueller et al. report virus attenuation by changes in codon bias. See, *Science*, 2008, 320:1784. See also WO/2008121992, WO/2006042156, Burns et al., *J Virology*, 2006, 80(7):3259 and Mueller et al., *J Virology*, 2006, 80(19):9687.

30 Luongo et al. report increased genetic and phenotypic stability of a live-attenuated respiratory syncytial virus vaccine candidate by reverse genetics. See *J. Virol.* 2012, 86(19):10792.

Dochow et al. report independent structural domains in paramyxovirus polymerase protein. *J Biol Chem*, 2012, 287:6878–91.

U.S. Patent 8,580,270 reports RSV F polypeptide sequences. U.S. Patent 7,951,384 reports that it contemplates a VLP RSV vaccine.

References cited herein are not an admission of prior art.

Any discussion of the prior art throughout the specification should in no way be
5 considered as an admission that such prior art is widely known or forms part of common general
knowledge in the field.

SUMMARY

According to a first aspect, the present invention provides an isolated recombinant
0 nucleic acid encoding nonstructural protein 1 (NS1) and optionally further comprising NS2 of a
wild-type human respiratory syncytial virus RSV, wherein the nucleotide sequence encoding
NS1 comprises SEQ ID NO:6 or SEQ ID NO:7 or a variant thereof having 95% or greater
sequence identity thereto.

According to a second aspect, the present invention provides a recombinant vector
5 comprising a nucleic acid of the invention.

According to a third aspect, the present invention provides an attenuated recombinant
RSV comprising a nucleic acid of the invention.

According to a fourth aspect, the present invention provides an expression system
comprising a vector of the invention or an attenuated recombinant RSV of the invention.

0 According to a fifth aspect, the present invention provides a vaccine comprising an
attenuated recombinant RSV of the invention.

According to a sixth aspect, the present invention provides use of the vaccine of the
invention in the manufacture of a medicament for the treatment of a subject who is younger than
6 months of age, under 1 year of age, born prematurely, has congenital heart or lung disease, is
25 having chemotherapy or has had a transplantation, or has been diagnosed with asthma,
congestive heart failure or chronic obstructive pulmonary disease, leukemia, elderly, or
HIV/AIDS.

According to a seventh aspect, the present invention provides an isolated recombinant
nucleic acid comprising an RSV genome OE1 of SEQ ID NO: 1, OE2 of SEQ ID NO: 2, OE3 of
30 SEQ ID NO: 3, OE4 of SEQ ID NO: 4 or variant with greater than 70, 71, 72, 73, 74, 75, 76, 77,
78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% or more
sequence identity thereto, wherein the genome comprises a nucleic acid of the invention.

According to an eighth aspect, the present invention provides a cell comprising the
recombinant vector of the invention.

35 According to a ninth aspect, the present invention provides a medicament comprising a

combination with the vaccine of the invention and motavizumab, palivizumab, or another humanized monoclonal antibody directed against an epitope in the A antigenic site of the F protein of RSV.

According to a tenth aspect, the present invention provides a method of treatment of a subject who is younger than 6 months of age, under 1 year of age, born prematurely, has congenital heart or lung disease, is having chemotherapy or has had a transplantation, or has been diagnosed with asthma, congestive heart failure or chronic obstructive pulmonary disease, leukemia, elderly, or HIV/AIDS comprising administering to the subject a therapeutically effective amount of the vaccine of the invention.

Unless the context clearly requires otherwise, throughout the description and the claims, the words "comprise", "comprising", and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of "including, but not limited to".

In certain embodiments, the disclosure relates to the polynucleotide sequences of respiratory syncytial virus (RSV). In certain embodiments, the disclosure relates to isolated or recombinant nucleic acids and polypeptides comprising desirable nucleic acid sequences and mutations disclosed herein. In certain embodiments, isolated or recombinant RSV comprising the nucleic acids and polypeptides disclosed herein (e.g., attenuated recombinant RSV) are also provided, as are immunogenic compositions including such nucleic acids, polypeptides, and RSV genomes that are suitable for use as vaccines. Attenuated or killed RSV containing these nucleic acids and mutation in the form of copied nucleic acids (e.g., cDNAs) are also contemplated.

In certain embodiments, this disclosure relates to isolated nucleic acids, recombinant respiratory syncytial virus (RSV) withcodon deoptimization, vaccines produced therefrom, and vaccination methods related thereto. In certain embodiments, the recombinant RSV comprises the genes NS1, NS2, N, P, M, SH, G, F, M2, and L of strain A2, line 19, or Long strain or variants thereof. In certain embodiments, the codon deoptimization is in the nonstructural genes NS1 and NS2and optionally in a gene G and optionally in a gene L. In further embodiments, the gene SH is deleted. In further embodiments, the gene F is mutated, e.g., an I to V mutation corresponding to residue 557 of RSV strain line19 F protein.

In certain embodiments, the disclosure relates to isolated nucleic acids encoding deoptimized genes NS1 and/or NS2and optionally the gene G and optionally the gene L of a wild-type human RSV or variant wherein the nucleotides are substituted such that a codon to produce Gly is GGT, a codon to produce Asp is GAT, a codon to produce Glu is GAA, a codon to produce His is CAT, a codon to produce Ile is ATA, a codon to produce Lys is AAA, a codon

to produce Leu is CTA, a codon to produce Asn is AAT, a codon to produce Gln is CAA, a codon to produce Val is GTA, or a codon to produce Tyr is TAT, or combinations thereof. In certain embodiments, a gene in the isolated nucleic acid further comprises a combination of at least two, three, four, five, six, seven, eight, nine, ten, or all of the individual codons. In certain embodiment, a gene in the isolated nucleic acid comprises at least 20, 30, 40, or 50 or more of the codons.

In certain embodiment, this disclosure relates to isolated nucleic acid as disclosed herein wherein the nucleotides are substituted such that a codon to produce Ala is GCG, a codon to produce Cys is TGT, a codon to produce Phe is TTT, a codon to produce Pro is CCG, a codon to produce Arg is CGT, a codon to produce Ser is TCG, or a codon to produce Thr is ACG, or combinations thereof. In certain embodiments, a gene containing the nucleic acid comprises a combination of at least two, three, four, five, six, seven, eight nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, or all of the individual codons. In certain embodiments, a gene in the isolated nucleic acid further comprises at least 20, 30, 40, or 50 or more of the codons.

10 In certain embodiments, the disclosure relates to nucleic acids disclosed herein encoding an NS1 having SEQ ID NO: 5

MGX¹NX²LSX³IKX⁴RLQNLX⁵X⁶NDEVALLKITCYX⁷DKLIX⁸LTNALAKAX⁹IHTIKL
NGIVFX¹⁰HVITSSX¹¹X¹²CPX¹³NX¹⁴IVVKSNSFTTMPX¹⁵LX¹⁶NGGYIX¹⁷EX¹⁸X¹⁹ELTH
CSQX²⁰NGX²¹X²²X²³DNCEIKFSX²⁴X²⁵LX²⁶DSX²⁷MTX²⁸YX²⁹X³⁰QX³¹SX³²LLGX³³DL
15 X³⁴X³⁵, wherein X¹-X³⁵ are any amino acid or
X¹ is S or C; X² is S or T; X³ is M or V; X⁴ is V or I; X⁵ is F or L; X⁶ is D or N; X⁷ is T or A; X⁸ is H, L, or Q; X⁹ is V or T; X¹⁰ is V or I; X¹¹ is D or E; X¹² is I, A, or V; X¹³ is N or D; X¹⁴ is N or S; X¹⁵ is V, I, or A; X¹⁶ is Q or R; X¹⁷ is W or any amino acid; X¹⁸ is M or L; X¹⁹ is M or I; X²⁰ is P or L; X²¹ is L or V; X²² is L, M, or I; X²³ is D or V; X²⁴ is K or R; 20 X²⁵ is K or R; X²⁶ is S or any amino acid; X²⁷ is T or V; X²⁸ is N or D; X²⁹ is M or I; X³⁰ is N or S; X³¹ is L or I; X³² is E or D; X³³ is F or L; X³⁴ is N or H; and X³⁵ is P or S or deleted.

25 In certain embodiments, the disclosure relates to nucleic acids disclosed herein encoding an NS1 of RSV as provided in NCBI Accession number NP_044589.1, NP_056856.1, P04544.1, AEQ63513.1, AFM55237.1, AFV32554.1, Q86306.1, AFV32528.1, AFM55248.1, AFM95358.1, AFV32568.1, ACY68428.1, CBW45413.1, ACO83290.1, AFM55347.1, CBW45433.1, AEQ63459.1, AFM55204.1, AFV32572.1, AFV32558.1, CBW45429.1, CBW45445.1, AFV32596.1, CBW45481.1, CBW47561.1, P24568.1, AAR14259.1, CBW45451.1, CBW45447.1, CBW45471.1, BAE96914.1, CBW45463.1, CBW45473.1, or CBW45467.1 or variants comprising one, two, or three 30 amino acid insertions, deletions, substitutions, or conserved substitutions.

In certain embodiments, the disclosure relates to an isolated nucleic acid comprising SEQ ID NO: 6 or SEQ ID NO: 7 or a sequence with 60%, 70%, 80%, 90%, 95% or greater sequence identity thereto.

In certain embodiments, the disclosure relates to nucleic acids disclosed herein encoding an NS2 having SEQ ID NO: 8,

5 MX¹TX²X³X⁴X⁵X⁶TX⁷QX⁸LX⁹ITDMRPX¹⁰SX¹¹X¹²X¹³X¹⁴IX¹⁵SLTX¹⁶X¹⁷IITHX¹⁸FIYLI
NX¹⁹ECIVX²⁰KLDEX²¹QATX²²X²³FLVNYEMX²⁴LLHX²⁵VGSX²⁶X²⁷YKKX²⁸TEYNTK
YGTFPMPIFIX²⁹HX³⁰GFX³¹ECIGX³²KPTKHTPIIX³³KYDLNP,
wherein X¹-X³³ are any amino acid or

10 X¹ is D or S; X² is T, A, or K; X³ is H, S, or N; X⁴ is N or P; X⁵ is D, G, or E; X⁶ is T or N;
X⁷ is P, M, Q, S, or A; X⁸ is R or G; X⁹ is M or I; X¹⁰ is L or M; X¹¹ is L, M, or I; X¹² is I,
D, or E; X¹³ is T or S; X¹⁴ is I or V; X¹⁵ is I or T; X¹⁶ is R or K; R¹⁷ is D or E; R¹⁸ is R or K;
R¹⁹ is H or N; X²⁰ is R or K; X²¹ is R or K; X²² is F or L; X²³ is T or A; X²⁴ is K or N; X²⁵ is
K or R; X²⁶ is T or A; X²⁷ is K or I; X²⁸ is T or S; X²⁹ is N or any amino acid; X³⁰ is D or G;
X³¹ is L or I; X³² is I or V; and X³³ is Y or H.

In certain embodiments, the disclosure relates to nucleic acids disclosed herein encoding an NS1 having an NS2 of RSV as provided in NCBI Accession number

15 NP_044590.1, NP_056857.1, CBW45420.1, AFM95337.1, CBW45416.1, CBW45430.1,
AFV32529.1, Q86305.1, AEQ63383.1, CBW45424.1, AFM55546.1, CBW45444.1,
P04543.2, AFM55326.1, AFM55425.1, AFM55381.1, AFM55458.1, AFM55216.1,
AAB59851.1, AEQ63372.1, AFM55337.1, CBW45426.1, AFV32515.1, AFV32519.1,
AAR14260.1, CBW47562.1, AFV32643.1, P24569.1, AFV32657.1 AFI25256.1,
20 CBW45480.1, AFV32605.1, AEQ63580.1, AFV32627.1, AFV32665.1, CBW45482.1,
CBW45478.1, CBW45462.1, AEQ63635.1, CBW45448.1, CBW45464.1, CBW45484.1, or
CBW45474.1 or variants comprising one, two or three amino acid insertions, deletions,
substitutions, or conserved substitutions.

In certain embodiments, the disclosure relates to an isolated nucleic acid comprising

25 SEQ ID NO: 9 or SEQ ID NO: 10 or a sequence with 60%, 70%, 80%, 90%, 95% or greater sequence identity thereto.

In certain embodiments, the disclosure relates to recombinant vectors comprising a nucleic acid disclosed herein.

In certain embodiments, the disclosure relates to an attenuated recombinant RSV

30 comprising a nucleic acid disclosed herein.

In certain embodiments, the disclosure relates to expression system comprising a vector disclosed herein or an attenuated recombinant RSV disclosed herein.

In certain embodiments, the disclosure relates to vaccines comprising an attenuated recombinant RSV disclosed herein.

In certain embodiments, the disclosure relates to methods of vaccination comprising administering an effective amount of a vaccine disclosed herein to a subject at risk of an RSV infection.

5 In certain embodiments, the subject is younger than 2 months or 6 months of age, under 1 year of age, born prematurely, have congenital heart or lung disease, having chemotherapy or a transplantation, or diagnosed with asthma, congestive heart failure or chronic obstructive pulmonary disease, leukemia, or HIV/AIDS.

10 In certain embodiments, vaccine is administered in combination with motavizumab, palivizumab, or another humanized monoclonal antibody directed against an epitope in the antigenic site II of the F protein of RSV.

15 In certain embodiments, the disclosure relates to vectors disclosed herein comprising a bacterial artificial chromosome (BAC), and a nucleic acid sequence comprising respiratory syncytial virus (RSV), and the BAC contains all genes that are essential for the generation of an infectious viral particle in a host cell. The nucleic acid sequence may be a viral genome or antigenome in operable combination with a regulatory element. Typically, the bacterial artificial chromosome comprises one or more genes selected from the group consisting of oriS, repE, parA, and parB genes of factor F in operable combination with a selectable marker, e.g., a gene that provides resistance to an antibiotic.

20 The nucleic acid sequence may be the genomic or antigenomic sequence of the virus which is optionally mutated as provided herein, e.g., RSV strain which is optionally mutated. In certain embodiments, the expression vector is a plasmid comprising MluI, Clal, BstBl, SacI restriction endonuclease cleavage sites and optionally an AvrII restriction endonuclease cleavage site outside the region of the wild-type viral sequence or outside the sequences that encode viral genes or outside the viral genome or antigenome. In certain 25 embodiments, the nucleic acid sequence further comprises a selectable marker or reporter gene in operable combination therewith, e.g., a gene that encodes a fluorescent protein.

30 In certain embodiments, the disclosure relates to isolated bacteria comprising one or more vectors disclosed herein, and other embodiments, the disclosure relates to an isolated cell comprising one or more vectors disclosed herein. In certain embodiments, the vector comprises an RSV antigenome and one or more vectors selected from the group consisting of: a vector encoding an N protein of RSV, a vector encoding a P protein of RSV, a vector encoding an L protein of RSV, and a vector encoding an M2-1 protein of RSV. Typically, the vector comprises a regulatory element, e.g., promoter, and the isolated eukaryotic cell expresses a nucleic acid or polypeptide that activates the regulatory element, e.g., encodes a

polypeptide that activates transcription downstream of the promoter. In certain embodiments, the promoter is T7, and the polypeptide that activates transcription downstream of the promoter is T7 RNA polymerase.

In certain embodiments, the disclosure relates to methods of generating respiratory syncytial virus (RSV) particles comprising inserting a vector with a BAC gene and a RSV antigenome into an isolated eukaryotic cell and inserting one or more vectors selected from the group consisting of: a vector encoding an N protein of RSV, a vector encoding a P protein of RSV, a vector encoding an L protein of RSV, and a vector encoding an M2-1 protein of RSV into the cell under conditions such that RSV virion is formed. Inserting a vector into a cell may occur by physically injecting, electroporating, or mixing the cell and the vector under conditions such that the vector enters the cell.

In certain embodiments, the disclosure relates to the stability of the line 19 F557 mutant virus compared to other strains, and val at 557 making RSV expressing line 19 F even more thermostable. Val at position 557 in other strains is also likely stabilizing; thus the 557 position is important for thermal stability. In certain embodiments, the disclosure contemplates other mutations in line 19 F or other RSV strains at position 557 (any amino acid, e.g., alanine, valine, isoleucine, leucine) in any F strain context, that improves thermostability of the RSV virus.

In certain embodiments, the disclosure contemplates RSV F polypeptide comprising an alanine, valine, or leucine at position 557, e.g., alanine or leucine in position 557 of SEQ ID NO: 17.

In certain embodiments, the disclosure relates to certain desirable sequence of RSV F polypeptides e.g., line 19 sequences comprising a valine at position 557, e.g., SEQ ID NO: 17, and recombinant nucleic acids encoding the same. In certain embodiments, the disclosure contemplates recombinant vectors comprising nucleic acids encoding these polypeptides and cells comprising said vectors.

In certain embodiments, the disclosure relates to immunogenic compositions comprising an immunologically effective amount of a recombinant respiratory syncytial virus (RSV), RSV polypeptide, RSV particle, RSV virus-like particle, and/or nucleic acid disclosed herein. In certain embodiments, the disclosure relates to methods for stimulating the immune system of an individual to produce a protective immune response against RSV. In certain embodiments, an immunologically effective amount of a RSV, polypeptide, and/or nucleic acid disclosed herein is administered to the individual in a physiologically acceptable carrier.

In certain embodiments, the disclosure relates to medicaments and vaccine products comprising nucleic acids disclosed herein for uses disclosed herein.

In certain embodiments, the disclosure relates to uses of nucleic acids or vectors disclosed herein for the manufacture of a medicament for uses disclosed herein.

5

BRIEF DESCRIPTIONS OF THE FIGURES

Figure 1 shows a table with the least used codons in human genes and in specific RSV strains.

Figure 2 shows growth data for kRSV-dNS1h in BEAS-2B (top) and Vero cell lines 10 (bottom). Growth curves of kRSV-A2 (open circle) and kRSV-dNSh (closed circle) in HEp-2 (A), Vero (B) and BEAS-2B (C) at 37 °C infected at MOI of 0.01, as well as in differentiated NHBE/ALI cells infected at MOI of 0.2 (D) or 2.0 (E).

Figure 3 shows data on viral load experiments using certain embodiments disclosed herein. Time course images for NHBE cells infection at MOI of 0.2, showing mKate2 15 fluorescence produced by the recombinant viruses. *P < 0.05

Figure 4 shows a gel after insertion of galK operon into BAC-RSV by recombineering. MluI digest. Lane 1, ladder marker. Mini-prep BAC DNAs (lanes 2 to 7). Lane 8, parental BAC-RSV “C2” clone. Lane 9, galK-containing plasmid. galK operon has a Mlu I restriction site that serves as a marker for introduction of galK by homologous 20 recombination.

Figure 5 shows a gel after deletion of galK operon from BAC-RSV by recombineering. MluI digest of galK-containing plasmid (lane 2), BAC mini-prep DNAs (lanes 3-7), and parental BAC-RSV clone C2 (lane 8).

Figures 6A-E schematically illustrates steps for creating a BAC-RSV. Three 25 plasmids with RSV segments are generated (see experimental); A) pKBS3 is cut at BstB1 and MluI sites to linearize, and is ligated to an oligonucleotide adapter providing pKBS5; B) pSynRSV#2 with SacI and Clal is cut and ligated to pKBS5 providing pKBS5-2; C) pSynRSV#3 with AvrII and MluI is cut and ligated to pKBS5_2 providing pKBS5_2_3; D) pSynRSV#1 with BstB1 and SacI is cut and ligated to pKBS5_2_3 providing 30 pKBS5_1_2_3. E). Recombineering is used to delete nucleotides between two Clal sites generating pSynRSV-line 19F.

Figure 7 shows data showing indicating the immunogenicity of an RSV strain with an F gene I557 to V mutation. Mice were infected with indicated doses of A2-K-line19F, A2-line19F-I557 V, or A2-K-A2GF and 29 days later challenged with RSV strain 12-35.

Lung viral load was measured day 4 post-challenge. The dotted line indicates the limit of detection.

Figure 8 shows data indicating the superior thermostability of RSV strains with an A2-line 19 F gene I557 to V mutation (SEQ ID NO:17). Viruses were incubated at indicated temperatures and viral titers were measured every day for 6 days. The results at 4°C are statistically significant between viruses ($P < 0.01$). The results at 37°C demonstrate the same phenotype.

Figure 9 illustrates an RSV sequence comparison of strain 19, I557 V mutation (SEQ ID NO:17) (Query) and the typical RSV strain 19 sequence (Sbjct).

Figure 10 illustrates an RSV sequence comparison of strain 19, I557 V mutation (SEQ ID NO:17) (Query) and sequence 61 from patent US 7,951,384 (Sbjct).

Figure 10 illustrates an RSV sequence comparison of strain 19, I557 V mutation (SEQ ID NO:17) (Query) sequence 12 from patent US 8,580,270 (Sbjct).

Figure 12 shows data on attenuation, efficacy, and immunogenicity of embodiments disclosed herein. (A) 6-8 week old BALB/c mice ($n = 5$ per group) were infected i.n. with 1.6×10^5 FFU of kRSV-A2 (open circle) or kRSV-dNSh (closed circle) and lung viral titer was assayed on days 1, 2, 4, 6, and 8 p.i.. Data represent one of two replicate experiments with similar results. * $P < .05$. (B) BALB/c mice were vaccinated i.n. with varying doses (10^5 FFU, 10^4 FFU, and 10^3 FFU) of kRSV-A2 (open circle) or kRSV-dNSh (closed circle), or mock-infected, and 100 days after vaccination, mice were challenged with 1.6×10^6 PFU RSV 12-35 strain. Lung peak viral loads were measured on day 4 after challenge. Each symbol represents one mouse. Dashed lines (A and B) denote the limit of detection for plaque assay. Titers below the limit of detection were assigned half the value of the limit of detection. (C) BALB/c mice ($n = 5$ per group) were mock-infected or infected with 10^5 FFU of either kRSV-A2 or kRSV-dNSh and serum nAb titers were measured at indicated days after infection. * $P < 0.05$.

Figure 13 shows data on vaccine efficacy for certain embodiments disclosed herein. 6-8 week old BALB/c mice ($n = 5$ per group) were mock-infected or vaccinated with varying indicated doses of kRSV-A2 (open circle) or kRSV-dNSh (closed circle). Mice were challenged 28 days later with (A) 2×10^6 PFU RSV A2-line19 strain or (B) 5×10^5 PFU RSV 12-35. Lung viral loads were measured day 4 after challenge. Each symbol represents one mouse. Dashed lines denote the limit of detection for plaque assay. Titers below the limit of detection were assigned half the value of the limit of detection.

Figure 14 shows data on the expression of NS1 and NS2 proteins during RSV infection in cell lines. HEp-2 (A), BEAS-2B (B) and Vero (C) cells were mock-infected or infected with either kRSV-A2, kRSV-dNSh, or kRSV-dNSv at MOI 5. Twenty hr p.i., NS1 and NS2 protein levels were analyzed by western blot and densitometry. Representative blots are shown on the left. Densitometry from 2-3 independent experiments is shown on the right. After normalizing to RSV N protein levels, NS1 and NS2 protein levels expressed by each virus were normalized to those during kRSV-A2 infection and expressed as percentage \pm SEM. Unfilled bars represent kRSV-A2, gray bars represent kRSV-dNSv, and black bars represent kRSV-dNSh.

10

DETAILED DESCRIPTION

Before the present disclosure is described in greater detail, it is to be understood that this disclosure is not limited to particular embodiments described, and as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose 15 of describing particular embodiments only, and is not intended to be limiting, since the scope of the present disclosure will be limited only by the appended claims.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Although any methods and materials similar or equivalent to those 20 described herein can also be used in the practice or testing of the present disclosure, the preferred methods and materials are now described.

All publications and patents cited in this specification are herein incorporated by reference as if each individual publication or patent were specifically and individually indicated to be incorporated by reference and are incorporated herein by reference to 25 disclose and describe the methods and/or materials in connection with which the publications are cited. The citation of any publication is for its disclosure prior to the filing date and should not be construed as an admission that the present disclosure is not entitled to antedate such publication by virtue of prior disclosure. Further, the dates of publication provided could be different from the actual publication dates that may need to be 30 independently confirmed.

As will be apparent to those of skill in the art upon reading this disclosure, each of the individual embodiments described and illustrated herein has discrete components and features which may be readily separated from or combined with the features of any of the other several embodiments without departing from the scope or spirit of the present

disclosure. Any recited method can be carried out in the order of events recited or in any other order that is logically possible.

5 Embodiments of the present disclosure will employ, unless otherwise indicated, techniques of immunology, medicine, organic chemistry, biochemistry, molecular biology, pharmacology, physiology, and the like, which are within the skill of the art. Such techniques are explained fully in the literature.

10 It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. In this specification and in the claims that follow, reference will be made to a number of terms that shall be defined to have the following meanings unless a contrary intention is apparent.

Prior to describing the various embodiments, the following definitions are provided and should be used unless otherwise indicated.

15 The terms "protein" and "polypeptide" refer to compounds comprising amino acids joined via peptide bonds and are used interchangeably.

The term "portion" when used in reference to a protein (as in "a portion of a given protein") refers to fragments of that protein. The fragments may range in size from four amino acid residues to the entire amino sequence minus one amino acid.

20 The term "chimera" when used in reference to a polypeptide refers to the expression product of two or more coding sequences obtained from different genes, that have been cloned together and that, after translation, act as a single polypeptide sequence. Chimeric polypeptides are also referred to as "hybrid" polypeptides. The coding sequences include those obtained from the same or from different species of organisms.

25 The term "homolog" or "homologous" when used in reference to a polypeptide refers to a high degree of sequence identity between two polypeptides, or to a high degree of similarity between the three-dimensional structure or to a high degree of similarity between the active site and the mechanism of action. In a preferred embodiment, a homolog has a greater than 60% sequence identity, and more preferably greater than 75% sequence identity, and still more preferably greater than 90% sequence identity, with a reference sequence.

30 As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more

(e.g., 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions.

The terms "variant" and "mutant" when used in reference to a polypeptide refer to an amino acid sequence that differs by one or more amino acids from another, usually related 5 polypeptide. The variant may have "conservative" changes, wherein a substituted amino acid has similar structural or chemical properties. One type of conservative amino acid substitutions refers to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is 10 serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups 15 are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine. More rarely, a variant may have "non-conservative" changes (e.g., replacement of a glycine with a tryptophan). Similar minor variations may also include amino acid deletions or insertions (in other words, additions), or both. Guidance in determining which and how many amino acid residues may be substituted, inserted or 20 deleted without abolishing biological activity may be found using computer programs well known in the art, for example, DNAStar software. Variants can be tested in functional assays. Preferred variants have less than 10%, and preferably less than 5%, and still more preferably less than 2% changes (whether substitutions, deletions, and so on).

The term "gene" refers to a nucleic acid (e.g., DNA or RNA) sequence that 25 comprises coding sequences necessary for the production of an RNA, or a polypeptide or its precursor (e.g., proinsulin). A functional polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence as long as the desired activity or functional properties (e.g., enzymatic activity, ligand binding, signal transduction, etc.) of the polypeptide are retained. The term "portion" when used in reference to a gene refers to 30 fragments of that gene. The fragments may range in size from a few nucleotides to the entire gene sequence minus one nucleotide. Thus, "a nucleotide comprising at least a portion of a gene" may comprise fragments of the gene or the entire gene.

The term "gene" also encompasses the coding regions of a structural gene and includes sequences located adjacent to the coding region on both the 5' and 3' ends for a

distance of about 1 kb on either end such that the gene corresponds to the length of the full-length mRNA. The sequences which are located 5' of the coding region and which are present on the mRNA are referred to as 5' non-translated sequences. The sequences which are located 3' or downstream of the coding region and which are present on the mRNA are 5 referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene which are transcribed into nuclear RNA (mRNA); introns may contain regulatory elements such as enhancers. Introns are 10 removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

In addition to containing introns, genomic forms of a gene may also include sequences located on both the 5' and 3' end of the sequences which are present on the RNA 15 transcript. These sequences are referred to as "flanking" sequences or regions (these flanking sequences are located 5' or 3' to the non-translated sequences present on the mRNA transcript). The 5' flanking region may contain regulatory sequences such as promoters and enhancers which control or influence the transcription of the gene. The 3' flanking region may contain sequences which direct the termination of transcription, posttranscriptional 20 cleavage and polyadenylation.

The term "heterologous gene" refers to a gene encoding a factor that is not in its natural environment (i.e., has been altered by the hand of man). For example, a heterologous gene includes a gene from one species introduced into another species. A heterologous gene also includes a gene native to an organism that has been altered in some 25 way (e.g., mutated, added in multiple copies, linked to a non-native promoter or enhancer sequence, etc.). Heterologous genes are distinguished from endogenous plant genes in that the heterologous gene sequences are typically joined to nucleotide sequences comprising regulatory elements such as promoters that are not found naturally associated with the gene for the protein encoded by the heterologous gene or with plant gene sequences in the 30 chromosome, or are associated with portions of the chromosome not found in nature (e.g., genes expressed in loci where the gene is not normally expressed).

The term "polynucleotide" refers to a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three, and usually more than ten. The exact size will depend on many factors, which in turn depends on the ultimate

function or use of the oligonucleotide. The polynucleotide may be generated in any manner, including chemical synthesis, DNA replication, reverse transcription, or a combination thereof. The term "oligonucleotide" generally refers to a short length of single-stranded polynucleotide chain usually less than 30 nucleotides long, although it may also be used 5 interchangeably with the term "polynucleotide."

The term "nucleic acid" refers to a polymer of nucleotides, or a polynucleotide, as described above. The term is used to designate a single molecule, or a collection of molecules. Nucleic acids may be single stranded or double stranded, and may include coding regions and regions of various control elements, as described below.

10 The term "a polynucleotide having a nucleotide sequence encoding a gene" or "a polynucleotide having a nucleotide sequence encoding a gene" or "a nucleic acid sequence encoding" a specified polypeptide refers to a nucleic acid sequence comprising the coding region of a gene or in other words the nucleic acid sequence which encodes a gene product. The coding region may be present in either a cDNA, genomic DNA or RNA form. When 15 present in a DNA form, the oligonucleotide, polynucleotide, or nucleic acid may be single-stranded (i.e., the sense strand) or double-stranded. Suitable control elements such as enhancers/promoters, splice junctions, polyadenylation signals, etc. may be placed in close proximity to the coding region of the gene if needed to permit proper initiation of transcription and/or correct processing of the primary RNA transcript. Alternatively, the 20 coding region utilized in the expression vectors of the present disclosure may contain endogenous enhancers/promoters, splice junctions, intervening sequences, polyadenylation signals, etc. or a combination of both endogenous and exogenous control elements.

The term "recombinant" when made in reference to a nucleic acid molecule refers to a nucleic acid molecule which is comprised of segments of nucleic acid joined together by 25 means of molecular biological techniques. The term "recombinant" when made in reference to a protein or a polypeptide refers to a protein molecule which is expressed using a recombinant nucleic acid molecule.

The terms "complementary" and "complementarity" refer to polynucleotides (i.e., a sequence of nucleotides) related by the base-pairing rules. For example, for the sequence 30 "A-G-T," is complementary to the sequence "T-C-A." Complementarity may be "partial," in which only some of the nucleic acids' bases are matched according to the base pairing rules. Or, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands. This is of particular

importance in amplification reactions, as well as detection methods which depend upon binding between nucleic acids.

The term "homology" when used in relation to nucleic acids refers to a degree of complementarity. There may be partial homology or complete homology (i.e., identity).

5 "Sequence identity" refers to a measure of relatedness between two or more nucleic acids or proteins, and is given as a percentage with reference to the total comparison length. The identity calculation takes into account those nucleotide or amino acid residues that are identical and in the same relative positions in their respective larger sequences. Calculations of identity may be performed by algorithms contained within computer programs such as

10 "GAP" (Genetics Computer Group, Madison, Wis.) and "ALIGN" (DNAStar, Madison, Wis.). A partially complementary sequence is one that at least partially inhibits (or competes with) a completely complementary sequence from hybridizing to a target nucleic acid is referred to using the functional term "substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target sequence may be

15 examined using a hybridization assay (Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (i.e., the hybridization) of a sequence which is completely homologous to a target under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low

20 stringency conditions require that the binding of two sequences to one another be a specific (i.e., selective) interaction. The absence of non-specific binding may be tested by the use of a second target which lacks even a partial degree of complementarity (e.g., less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

25 The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence", "sequence identity", "percentage of sequence identity", and "substantial identity". A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA sequence given in a sequence listing or

30 may comprise a complete gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two

polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide 5 positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a 10 comparison window may be conducted by the local homology algorithm of Smith and Waterman (Smith and Waterman, *Adv. Appl. Math.* 2: 482 (1981)) by the homology alignment algorithm of Needleman and Wunsch (Needleman and Wunsch, *J. Mol. Biol.* 48:443 (1970)), by the search for similarity method of Pearson and Lipman (Pearson and Lipman, *Proc. Natl. Acad. Sci. (U.S.)* 85:2444 (1988)), by computerized implementations of 15 these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, Wis.), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a 20 nucleotide-by-nucleotide basis) over the window of comparison.

In certain embodiments, term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of 25 matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity.

In certain embodiments, sequence "identity" refers to the number of exactly matching amino acids (expressed as a percentage) in a sequence alignment between two 30 sequences of the alignment calculated using the number of identical positions divided by the greater of the shortest sequence or the number of equivalent positions excluding overhangs wherein internal gaps are counted as an equivalent position. For example the polypeptides GGGGGG and GGGGT have a sequence identity of 4 out of 5 or 80%. For example, the polypeptides GGGPPP and GGGAPPP have a sequence identity of 6 out of 7 or 85%. In

certain embodiments, any recitation of sequence identity expressed herein may be substituted for sequence similarity. Percent "similarity" is used to quantify the similarity between two sequences of the alignment. This method is identical to determining the identity except that certain amino acids do not have to be identical to have a match. Amino 5 acids are classified as matches if they are among a group with similar properties according to the following amino acid groups: Aromatic - F Y W; hydrophobic-A V I L; Charged positive: R K H; Charged negative - D E; Polar - S T N Q.

The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 10 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or 15 additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence, for example, as a segment of the full-length sequences of the compositions claimed in the present disclosure.

When used in reference to a double-stranded nucleic acid sequence such as a cDNA or genomic clone, the term "substantially homologous" refers to any probe that can 20 hybridize to either or both strands of the double-stranded nucleic acid sequence under conditions of low to high stringency as described above.

When used in reference to a single-stranded nucleic acid sequence, the term "substantially homologous" refers to any probe that can hybridize (i.e., it is the complement of) the single-stranded nucleic acid sequence under conditions of low to high stringency as 25 described above.

The terms "in operable combination", "in operable order" and "operably linked" refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in 30 such a manner so that a functional protein is produced.

The term "regulatory element" refers to a genetic element which controls some aspect of the expression of nucleic acid sequences. For example, a promoter is a regulatory element which facilitates the initiation of transcription of an operably linked coding region.

Other regulatory elements are splicing signals, polyadenylation signals, termination signals, etc.

Transcriptional control signals in eukaryotes comprise "promoter" and "enhancer" elements. Promoters and enhancers consist of short arrays of DNA sequences that interact 5 specifically with cellular proteins involved in transcription (Maniatis, et al., *Science* 236:1237, 1987). Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in yeast, insect, mammalian and plant cells. Promoter and enhancer elements have also been isolated from viruses and are found in prokaryotes. The selection of a particular promoter and enhancer depends on the cell type used to express 10 the protein of interest. Some eukaryotic promoters and enhancers have a broad host range while others are functional in a limited subset of cell types (for review, see Voss, et al., *Trends Biochem. Sci.*, 11:287, 1986; and Maniatis, et al., *supra* 1987).

The terms "promoter element," "promoter," or "promoter sequence" as used herein, refer to a DNA sequence that is located at the 5' end (i.e. precedes) the protein coding 15 region of a DNA polymer. The location of most promoters known in nature precedes the transcribed region. The promoter functions as a switch, activating the expression of a gene. If the gene is activated, it is said to be transcribed, or participating in transcription. Transcription involves the synthesis of mRNA from the gene. The promoter, therefore, serves as a transcriptional regulatory element and also provides a site for initiation of 20 transcription of the gene into mRNA.

Promoters may be tissue specific or cell specific. The term "tissue specific" as it applies to a promoter refers to a promoter that is capable of directing selective expression of a nucleotide sequence of interest to a specific type of tissue (e.g., seeds) in the relative absence of expression of the same nucleotide sequence of interest in a different type of 25 tissue (e.g., leaves). Tissue specificity of a promoter may be evaluated by, for example, operably linking a reporter gene to the promoter sequence to generate a reporter construct, introducing the reporter construct into the genome of a plant such that the reporter construct is integrated into every tissue of the resulting transgenic plant, and detecting the expression of the reporter gene (e.g., detecting mRNA, protein, or the activity of a protein encoded by 30 the reporter gene) in different tissues of the transgenic plant. The detection of a greater level of expression of the reporter gene in one or more tissues relative to the level of expression of the reporter gene in other tissues shows that the promoter is specific for the tissues in which greater levels of expression are detected. The term "cell type specific" as applied to a promoter refers to a promoter which is capable of directing selective expression of a

nucleotide sequence of interest in a specific type of cell in the relative absence of expression of the same nucleotide sequence of interest in a different type of cell within the same tissue. The term "cell type specific" when applied to a promoter also means a promoter capable of promoting selective expression of a nucleotide sequence of interest in a region within a

5 single tissue. Cell type specificity of a promoter may be assessed using methods well known in the art, e.g., immunohistochemical staining. Briefly, tissue sections are embedded in paraffin, and paraffin sections are reacted with a primary antibody which is specific for the polypeptide product encoded by the nucleotide sequence of interest whose expression is controlled by the promoter. A labeled (e.g., peroxidase conjugated) secondary antibody

10 which is specific for the primary antibody is allowed to bind to the sectioned tissue and specific binding detected (e.g., with avidin/biotin) by microscopy.

Promoters may be constitutive or regulatable. The term "constitutive" when made in reference to a promoter means that the promoter is capable of directing transcription of an operably linked nucleic acid sequence in the absence of a stimulus (e.g., heat shock, chemicals, light, etc.). Typically, constitutive promoters are capable of directing expression of a transgene in substantially any cell and any tissue.

In contrast, a "regulatable" or "inducible" promoter is one which is capable of directing a level of transcription of an operably linked nucleic acid sequence in the presence of a stimulus (e.g., heat shock, chemicals, light, etc.) which is different from the level of

20 transcription of the operably linked nucleic acid sequence in the absence of the stimulus.

The enhancer and/or promoter may be "endogenous" or "exogenous" or "heterologous." An "endogenous" enhancer or promoter is one that is naturally linked with a given gene in the genome. An "exogenous" or "heterologous" enhancer or promoter is one that is placed in juxtaposition to a gene by means of genetic manipulation (i.e., molecular

25 biological techniques) such that transcription of the gene is directed by the linked enhancer or promoter. For example, an endogenous promoter in operable combination with a first gene can be isolated, removed, and placed in operable combination with a second gene, thereby making it a "heterologous promoter" in operable combination with the second gene. A variety of such combinations are contemplated (e.g., the first and second genes can be

30 from the same species, or from different species).

Efficient expression of recombinant DNA sequences in eukaryotic cells typically requires expression of signals directing the efficient termination and polyadenylation of the resulting transcript. Transcription termination signals are generally found downstream of the polyadenylation signal and are a few hundred nucleotides in length. The term "poly(A)

site" or "poly(A) sequence" as used herein denotes a DNA sequence which directs both the termination and polyadenylation of the nascent RNA transcript. Efficient polyadenylation of the recombinant transcript is desirable, as transcripts lacking a poly(A) tail are unstable and are rapidly degraded. The poly(A) signal utilized in an expression vector may be

5 "heterologous" or "endogenous." An endogenous poly(A) signal is found naturally at the 3' end of the coding region of a given gene in the genome. A heterologous poly(A) signal is one which has been isolated from one gene and positioned 3' to another gene. A commonly used heterologous poly(A) signal is the SV40 poly(A) signal. The SV40 poly(A) signal is contained on a 237 bp BamHI/BclI restriction fragment and directs both termination and

10 polyadenylation.

The term "vector" refers to nucleic acid molecules that transfer DNA segment(s) from one cell to another. The term "vehicle" is sometimes used interchangeably with "vector."

The terms "expression vector" or "expression cassette" refer to a recombinant

15 nucleic acid containing a desired coding sequence and appropriate nucleic acid sequences used for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences used for expression in prokaryotes typically include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and

20 polyadenylation signals.

The term "host cell" refers to any cell capable of replicating and/or transcribing and/or translating a heterologous gene. Thus, a "host cell" refers to any eukaryotic or prokaryotic cell (e.g., bacterial cells such as *E. coli*, yeast cells, mammalian cells, avian cells, amphibian cells, plant cells, fish cells, and insect cells), whether located *in vitro* or *in vivo*. For example, host cells may be located in a transgenic animal.

The term "selectable marker" refers to a gene which encodes an enzyme having an activity that confers antibiotic or drug resistance upon the cell in which the selectable marker is expressed, or which confers expression of a trait which can be detected (e.g., luminescence or fluorescence). Selectable markers may be "positive" or "negative."

30 Examples of positive selectable markers include the neomycin phosphotransferase (NPTII) gene which confers resistance to G418 and to kanamycin, and the bacterial hygromycin phosphotransferase gene (hyg), which confers resistance to the antibiotic hygromycin. Negative selectable markers encode an enzymatic activity whose expression is cytotoxic to the cell when grown in an appropriate selective medium. For example, the HSV-tk gene is

commonly used as a negative selectable marker. Expression of the HSV-tk gene in cells grown in the presence of gancyclovir or acyclovir is cytotoxic; thus, growth of cells in selective medium containing gancyclovir or acyclovir selects against cells capable of expressing a functional HSV TK enzyme.

5 The term "reporter gene" refers to a gene encoding a protein that may be assayed. Examples of reporter genes include, but are not limited to, modified katushka, mkate and mkate2 (See, e.g., Merzlyak et al., *Nat. Methods*, 2007, 4, 555–557 and Shcherbo et al., *Biochem. J.*, 2008, 418, 567–574), luciferase (See, e.g., deWet et al., *Mol. Cell. Biol.* 7:725 (1987) and U.S. Pat Nos., 6,074,859; 5,976,796; 5,674,713; and 5,618,682; all of which are 10 incorporated herein by reference), green fluorescent protein (e.g., GenBank Accession Number U43284; a number of GFP variants are commercially available from ClonTech Laboratories, Palo Alto, Calif.), chloramphenicol acetyltransferase, beta-galactosidase, alkaline phosphatase, and horse radish peroxidase.

15 The term "wild-type" when made in reference to a gene refers to a gene which has the characteristics of a gene isolated from a naturally occurring source. The term "wild-type" when made in reference to a gene product refers to a gene product which has the characteristics of a gene product isolated from a naturally occurring source. The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is 20 present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designated the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" when made in reference to a gene or to a gene product refers, 25 respectively, to a gene or to a gene product which displays modifications in sequence and/or functional properties (i.e., altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product.

30 The term "antisense" or "antigenome" refers to a nucleotide sequence whose sequence of nucleotide residues is in reverse 5' to 3' orientation in relation to the sequence of nucleotide residues in a sense strand. A "sense strand" of a DNA duplex refers to a strand in a DNA duplex which is transcribed by a cell in its natural state into a "sense mRNA."

Thus an "antisense" sequence is a sequence having the same sequence as the non-coding strand in a DNA duplex.

The term "isolated" refers to a biological material, such as a virus, a nucleic acid or a protein, which is substantially free from components that normally accompany or interact with it in its naturally occurring environment. The isolated material optionally comprises material not found with the material in its natural environment, e.g., a cell. For example, if the material is in its natural environment, such as a cell, the material has been placed at a location in the cell (e.g., genome or genetic element) not native to a material found in that environment. For example, a naturally occurring nucleic acid (e.g., a coding sequence, a promoter, an enhancer, etc.) becomes isolated if it is introduced by non-naturally occurring means to a locus of the genome (e.g., a vector, such as a plasmid or virus vector, or amplicon) not native to that nucleic acid. Such nucleic acids are also referred to as "heterologous" nucleic acids. An isolated virus, for example, is in an environment (e.g., a cell culture system, or purified from cell culture) other than the native environment of wild-type virus (e.g., the nasopharynx of an infected individual).

An "immunologically effective amount" of RSV is an amount sufficient to enhance an individual's (e.g., a human's) own immune response against a subsequent exposure to RSV. Levels of induced immunity can be monitored, e.g., by measuring amounts of neutralizing secretory and/or serum antibodies, e.g., by plaque neutralization, complement fixation, enzyme-linked immunosorbent, or microneutralization assay.

A "protective immune response" against RSV refers to an immune response exhibited by an individual (e.g., a human) that is protective against serious lower respiratory tract disease (e.g., pneumonia and/or bronchiolitis) when the individual is subsequently exposed to and/or infected with wild-type RSV.

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Recombinant respiratory syncytial virus (RSV) with codon usage silent mutations in the nonstructural genes

Live-attenuated RSV vaccine candidates have two major hurdles, suboptimal immunogenicity in infants and suboptimal stability that leads to genetic reversion towards wild-type and shedding of revertants by vaccinees. The viral nonstructural (NS) proteins, NS1 and NS2, are unique and inhibit type I interferon and T cell responses. Mutating NS1/NS2 for vaccine enhances immunogenicity. However, previously developed NS1 and NS1/NS2 deletion/null mutant recombinant RSV strains are over-attenuated, and the NS2 null mutant is under-attenuated in vivo.

Mutants disclosed herein overcome the limitations of over-attenuation and instability. Mutants were generated with partial NS1 and NS2 function to bridge the attenuation-immunogenicity gap for a pediatric vaccine. Gene synthesis and the RSV BAC rescue system was used to generate NS1/NS2 mutants by altering codon usage across the

5 NS1 and NS2 genes. Codon de-optimization reduces translation efficiency by multiple mechanisms (e.g., tRNA concentration and mRNA structure). One mutant disclosed herein (“dNSh”) has 84/420 nt of NS1 mutated and 82/375 nt of NS2 mutated, reducing human codon preference without altering the amino acid sequences. This virus produces approximately 25% of wt NS1 levels, 25% of wt NS2 levels, 100% of wt nucleoprotein

10 levels, and replicates like wt virus in Vero cells, the cell line commonly used to produce live attenuated RSV under GMP conditions (Fig. 2). In addition to reducing NS expression, this approach likely solves the genetic stability problem because there are too many mutations for reversion.

In certain embodiments, the disclosure relates to a vaccine, recombinant RSV genome, or an isolated recombinant nucleic acid encoding RSV NS1, NS2, N, P, M, G, F, M2-1, M2-2, and L genes comprising codon-deoptimization of the NS1 and NS2 genes, wherein codon-deoptimization is configured such that at least one codon to produce Gly is GGT, a codon to produce Asp is GAT, at least one codon to produce Glu is GAA, at least one codon to produce His is CAT, at least one codon to produce Ile is ATA, at least one codon to produce Lys is AAA, at least one codon to produce Leu is CTA, at least one codon to produce Asn is AAT, at least one codon to produce Gln is CAA, at least one codon to produce Val is GTA, or at least one codon to produce Tyr is TAT, wherein in greater than 25% of the Asp, Glu, His, Ile, Lys, Leu, Asn, Gln, Val, and Tyr amino acids are codon-deoptimized. In certain embodiments, greater than 75% of the amino acids are codon-deoptimized as compared to wild-type sequences, e.g., RSV A2 line 19.

In certain embodiments, the NS1 gene comprises (SEQ ID NO: 6) or variant thereof with greater than 70, 80, 90, 95, 97, 98, or 99% or more sequence identity thereto.

In certain embodiments, the NS2 gene comprises (SEQ ID NO: 9) or variant thereof with greater than 70, 80, 90, 95, 97, 98, or 99% or more sequence identity thereto.

30 In certain embodiments, the RSV small hydrophobic (SH) glycoprotein gene is deleted.

In certain embodiments, the nucleic acid has further codon-deoptimization of the G gene, wherein codon-deoptimization is configured such that at least one codon to produce Gly is GGT, a codon to produce Asp is GAT, at least one codon to produce Glu is GAA, at

least one codon to produce His is CAT, at least one codon to produce Ile is ATA, at least one codon to produce Lys is AAA, at least one codon to produce Leu is CTA, at least one codon to produce Asn is AAT, at least one codon to produce Gln is CAA, at least one codon to produce Val is GTA, or at least one codon to produce Tyr is TAT, wherein in greater than 5 25% of the Asp, Glu, His, Ile, Lys, Leu, Asn, Gln, Val, and Tyr amino acids are codon-deoptimized.

In certain embodiments, the G gene comprises SEQ ID NO: 18

ATGTCGAAAAACAAAGACCAACGTACCGCGAAGACGCTAGAACGTACCTGGGA
 TACTCTAAATCATTACTATTATCGTCGTGCCTATATAAGCTAAATCTTAAA
 10 TCGGTAGCACAAATAACACTATCCATACTGGCGATAATAATCTGACTTCGCTT
 ATAATAGCAGCGATCATATTATAGCCTCGCGAACCATAAAGTCACGCCAACG
 ACTGCGATCATACAAGATGCGACATCGCAGATAAAGAATAACAACGCCAACGTA
 CCTAACCCAAAATCCTCAACTTGGTATCTGCCCTCGAATCCGTCTGAAATAAC
 ATCGCAAATCACGACCATACTAGCGTCAACGACACCAGGAGTAAAGTCGACCC
 15 TACAATCCACGACAGTAAAGACGAAAAACACGACAACGACTCAAACGCAACCC
 TCGAAGCCGACCACGAAACAAACGCCAAAATAAACCAACCGAGCAAACCGAATAA
 TGATTTCACTTGAAGTATTCAATTGTACCCCTGTAGCATATGTAGCAATAAT
 CCAACGTGCTGGCGATCTGTAAAAGAATAACCGAACAAAAACCGGGAAAAAA
 AACACGACCAACCCACGAAAAACCAACGCTAAAACAACGAAAAAGAT
 20 CCCAAACCGCAAACCACGAAATCAAAAGAAGTACCCACGACCAAACCCACGGA
 AGAGCCGACCATAAACACGACCAAAACGAACATAATAACTACGCTACTCACGT
 CCAATACCACGGAAATCCGGAACTCACGAGTCAAATGGAAACGTTCACTCG
 ACTTCGTCCGAAGGTAATCCATCGCCTCGCAAGTCTCGACAACGTCCGAATAC
 CCGTCACAACCGTCATGCCACCGAACACGCCACGTCAGTAG or variant thereof
 25 with greater than 70, 80, 90, 95, 97, 98, or 99% or more sequence identity thereto.

In certain embodiments, the G gene comprises SEQ ID NO: 19

ATGTCGAAAAATAAAGACCAACGTACGGCGAAGACGCTAGAACGTACCTGGGA
 TACGCTAAATCATTACTATTATATCGTCGTGCCTATATAAAACTAAATCTTAAA
 TCGGTAGCGCAAATAACACTATCGATACTGGCGATAATAATATCGACTTCGCTA
 30 ATAATAGCAGCGATAATATTATAGCCTCGCGAACATCAAAGTCACGCCACG
 ACTGCGATAATACAAGATGCGACATCGCAAATAAGAATAACGACGCCAACGTA
 TCTAACCCAAAATCCGCAACTTGGTATATGCCCTCGAACCGTGGAAATAAC
 ATCGCAAATAACGACCATACTAGCGTCGACGACACCAGGAGTAAAGTCGACGC
 TACAATCCACGACGGTAAAGACGAAAAATACGACAAACGACGCCAACCG

TCGAAACCGACCACGAAACAAACGTAAAATAACCACCGTCGAAACCGAATAA
TGATTTCACTTGAAGTATTAAATTGTACCCCTGTCGATATGTAGCAATAAT
CCGACGTGCTGGCGATATGTAAAAGAATACCGAATAAAAACCGGGAAAAAA
AACGACGACCAAACCGACGAAAAAACCAACGCTAAAACAACGAAAAAGAT
5 CCGAAACCGCAAACCACGAAATCGAAAGAAGTACCCACGACGAAACCCACGG
AAGAACCGACCATAAAATACGACCAAAACGAATATAACTACGCTACTAACG
TCCAATACGACGGAAATCCGAACTAACGAGTCAAATGGAAACGTTTCATT
GACTTCGTCGGAAGGTAATCCATGCCGTCGCAAGTCTCGACGACTTCCGAATA
TCCGTCACAACCGTCGCCACCGAATACGCCACGTCAATAG or variant thereof
10 with greater than 70, 80, 90, 95, 97, 98, or 99% or more sequence identity thereto.

In certain embodiments, the G gene comprises SEQ ID NO: 20

ATGTCGAAAAATAAGATCAACGTACGGCGAAACGCTAGAACGTACGTGGGA
TACGCTAAATCATCTACTATTATATCGTCGTCTATATAAACTAAATCTAAAA
TCGGTAGCGCAAATAACGCTATCGATACTAGCGATAATAATATCGACTTCGCTA
15 ATAATAGCGCGATAATATTATAGCGTCGGCGAATCATAAAAGTAACGCCGAC
GACGGCGATAATACAAGATGCGACTTCGCAAATAAAAATACGACGCCGACGT
ATCTAACGCAAATCCGCAACTAGGTATATGCCGTCGAATCCGTCGGAAATAA
CGTCGCAAATAACGACGATACTAGCGTCGACGACGCCGGTGTAAAATCGACG
CTACAATCGACGACGGTAAAACGAAAAATACGACGACGACGACGCAAACGCAACC
20 GTCGAAACCGACGACGAAACAAACGTAAAATAACCGCCGTCGAAACCGAATA
ATGATTTCACTTGAAGTATTAAATTGTACCGTGTTCGATATGTTCGAATAA
TCCGACGTGTTGGCGATATGTAAACGTATACCGAATAAAAACCGGGTAAAAA
AAACGACGACGAAACCGACGAAAAACCGACGCTAAAACGACGAAAAAGA
TCCGAAACCGCAAACGACGAAATCGAAAGAAGTACCGACGACGACGAAACCGACG
25 GAAGAACCGACGATAATACGACGAAACGAATATAAAACGACGCTACTAAC
GTCGAATACGACGGTAATCCGAACTAACGTCGCAAATGGAAACGTTTCATT
GACtTCGTCGGAAGGTAATCCGTCGCCGTCGCAAGTATCGACGACtTCGGAATAT
CCGTCGCAAACCGTCGCCCGAATACGCCGCGTCAATAG or variant thereof
with greater than 70, 80, 90, 95, 97, 98, or 99% or more sequence identity thereto.

30 In certain embodiments, F gene encodes a valine at position 557 and lysine at position 66. In certain embodiments, F gene encodes a valine at position 557 and the F gene comprises a sequence that encodes one or more of the follow amino acid sequences F gene comprises two, three, four, five or all of the follow amino acid sequences
TTNIMITTIIIVIIVILLSLIAVGLLYCK (SEQ ID NO: 11),

ARSTPVPILKANAITTILAAVTFCFA (SEQ ID NO: 12), AVTFCFASSQNITEEFYQST (SEQ ID NO: 13), QSTCSAVSKGYLSALRTGWYTSVITIELSNIKK (SEQ ID NO: 14), IKK NKCGNTDAKVKLMKQELDKYKNAV (SEQ ID NO: 15), and FPQAEKCKVQSNRVFC DTMYSLLPSEVNLCNV (SEQ ID NO: 16).

5 In certain embodiments, the F gene comprises two, three, four, five or all of the follow amino acid sequences (SEQ ID NO: 11), (SEQ ID NO: 12), (SEQ ID NO: 13), (SEQ ID NO: 14), (SEQ ID NO: 15), and (SEQ ID NO: 16).

10 In certain embodiments, the F gene encodes a valine at position 557 and the F gene encodes one or more of the follow amino acids: asparagine at position 8, phenylalanine at position 20, serine at position 35, lysine at position 66, methionine at position 79, lysine at position 124, arginine at position 191, arginine at position 213, glutamic acid at position 354, lysine at position 357, tyrosine at position 371, valine at position 384, asparagine at position at 115, and threonine at position 523.

15 In certain embodiments, the F gene encodes a valine at position 557 and lysine at position 66 and methionine at position 79.

In certain embodiments, the F gene encodes a valine at position 557 and lysine at position 66 and arginine at position 191.

In certain embodiments, the F gene encodes a valine at position 557, lysine at position 66, arginine at position 191, and lysine at position 357.

20 In certain embodiments, the F gene encodes a valine at position 557, lysine at position 66, methionine at position 79, and asparagine at position at 115.

In certain embodiments, the F gene encodes SEQ ID NO: 17

MELPILKANAITTILAAVTFCFASSQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITI
ELSNIKKNKCGNTDAKVKLMKQELDKYKNAVTELQLLMQSTPAANNRARRELPRF
25 MNYTLNNTKKTNTVTLSSKKRKRFLGFLGVGSIAASGIAVSKVLHLEGEVNKIKSA
LLSTNKAVVSLNSNGVSVLTSRVLDLKNYIDKQLLPIVNKQSCRISNIETVIEFQQKNN
RLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQS
YSIMSIKEEVLAYVVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLRTDRGWY
CDNAGSVSFFPQAEKCKVQSNRVFCDTMYSLLPSEVNLCNVDFNPKYDCKIMTS
30 KTDVSSSVITSLGAIVSCYGYKTKCTASNKNRGIKTFNSNGCDYVSNKGVDIVTSGNT
LYYVNKQEGKSLYVKGEPIINFYDPLVFPSEFDASISQVNEKINQSLAFIRKSDELL
HNVNAGKSTTNIMITIIIVILLSLIAVGLLLICKARSTPVTLSKDQLSGINNIAFSN
or variants that contain one, two, three, four, five, six, seven, eight, nine, or ten amino acid

substitutions provided F gene encodes a valine at position 557. In certain embodiments, the amino acid substitutions are conservative substitutions.

5 In certain embodiments, the disclosure relates to an isolated recombinant nucleic acid comprising an F gene encoding (SEQ ID NO: 17) or variants that contains one or two amino acid substitutions provided F gene encodes a valine at position 557 and lysine at position 66.

10 In certain embodiments, the F gene encodes a valine at position 557 and the F gene encodes one or more of the follow amino acids: asparagine at position 8, phenylalanine at position 20, serine at position 35, lysine at position 66, methionine at position 79, lysine at position 124, arginine at position 191, arginine at position 213, glutamic acid at position 354, lysine at position 357, tyrosine at position 371, valine at position 384, asparagine at position at 115, and threonine at position 523.

15 In certain embodiments, the F gene encodes a valine at position 557 and lysine at position 66.

15 In certain embodiments, the F gene encodes a valine at position 557 and lysine at position 66 and methionine at position 79.

In certain embodiments, the F gene encodes a valine at position 557, lysine at position 66, arginine at position 191, and lysine at position 357.

20 In certain embodiments, the F gene encodes a valine at position 557, lysine at position 66, methionine at position 79, and asparagine at position at 115.

In certain embodiments, the disclosure relates to a recombinant vector comprising a nucleic acid disclosed herein. In certain embodiments, the disclosure relates to a cell comprising the recombinant vector, recombinant RSV, or attenuated recombinant RSV disclosed herein.

25 In certain embodiments, the disclosure relates to an F gene encoding (SEQ ID NO: 17) or variants that contains one amino acid substitutions provided F gene encodes a valine at position 557.

In certain embodiments, the disclosure relates to an F gene encoding
MELPILKANAITTILAAVTFCFASSQNITEEFYQSTCSAVSKGYLSALRTGWYTSVITI
30 ELSNIKENCNGTDAKVKLMQELDKYKNAVTELQLLMQSTPAANNRARRELPRF
MNYTLNNTKKTNTVTLSSKKRKRFLGFLGVGSAIASGIAVSKVLHLEGEVNKIKSA
LLSTNKAVVSLNSNGSVLTSRVLDLKNYIDKQLLPIVNKQSCRISNIETVIEFQQKNN
RLLEITREFSVNAGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNVQIVRQQS
YSIMSIKEEVLAYVQLPLYGVIDTPCWKLHTSPLCTTNTKEGSNICLRTDRGWY

CDNAGSVSFFPQAEKCKVQSNRVFCDTMYSLLPSEVNLCNVDIFNPKYDCKIMTS
KTDVSSSVITSLGAIVSCYGKTKCTASNKNRGIIKTFNSNGCDYVSNKGVDTSVGNT
LYYVNKQEGKSLYVKGEPIINFYDPLVFPSEFDASISQVNEKINQLAFIRKSDELL
HNVNAGKSTTNIMITTIIIVILLSLIAVGLLYCKARSTPVTLSKDQLSGINNIAFSN
5 (SEQ ID NO: 21). In certain embodiments, the F gene encodes a valine at position 557 and
glutamic acid at position 66 and arginine at position 191.

In certain embodiments, the disclosure relates to recombinant polypeptides
comprising an RSV F protein sequence disclosed herein. In certain embodiments, the
disclosure relates to virus particles or virus like particles produced by recombinant methods
10 comprising a RSV F protein sequence disclosed herein.

In certain embodiments, the disclosure relates to an isolated recombinant nucleic
acid comprising an RSV genome OE1 of SEQ ID NO: 1 or variant with greater than 70, 71,
72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95,
96, 97, 98, or 99% or more sequence identity thereto.

15 In certain embodiments, the disclosure relates to an isolated recombinant nucleic
acid comprising an RSV genome OE2 of SEQ ID NO: 2 or variant with greater than 70, 71,
72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95,
96, 97, 98, or 99% or more sequence identity thereto.

20 In certain embodiments, the disclosure relates to an isolated recombinant nucleic
acid comprising an RSV genome OE3 of SEQ ID NO: 3 or variant with greater than 70, 71,
72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95,
96, 97, 98, or 99% or more sequence identity thereto.

25 In certain embodiments, the disclosure relates to an isolated recombinant nucleic
acid comprising an RSV genome OE4 of SEQ ID NO: 4 or variant with greater than 70, 71,
72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95,
96, 97, 98, or 99% or more sequence identity thereto.

30 In certain embodiments, the disclosure contemplates isolated recombinant nucleic
acids comprising RSV genomes OE1, OE2, OE3, and OE4, wherein one or both of the NS1
gene and NS2 gene are deleted.

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Cultivating RSV in a bacterial artificial chromosome

Cultivating RSV in *E. coli* bacteria may be accomplished by utilizing a bacterial
artificial chromosome (BAC). A BAC is disclosed that contains the complete antigenomic
sequence of respiratory syncytial virus (RSV) strain A2 except the F gene, which is the

antigenomic sequence of RSV strain line 19. Along with helper plasmids, it can be used in the reverse genetics system for the recovery of infectious virus. The antigenome sequence on the plasmid can be mutated prior to virus recovery to generate viruses with desired mutations.

5 The plasmid is an improvement on current RSV antigenomic plasmids for several reasons. Each RSV gene is flanked by restriction endonuclease cleavage sites to allow for easy manipulation of any gene. As a basis for viral mutagenesis, this plasmid may be used to design attenuated viruses for use in vaccines. An extra gene encoding the monomeric katushka 2, mKate2, protein has been included in the antigenome prior to the first RSV 10 gene. The mKate2 protein is a far-red fluorescent protein which would be expressed in concert with the other RSV genes and would serve as visual evidence of virus replication. Changes have also been made to the ribozyme sequences that flank the RSV antigenome and play a role in the production of infectious virus through reverse genetics.

The disclosed vectors allow for efficient mutagenesis through recombineering. This 15 mutagenesis method requires little to no ligation cloning, but relies on the recombination machinery present in bacteria harboring certain genes from a bacteriophage. Because RSV cDNAs are often unstable in mid-to-high copy number cloning vectors within bacteria predominantly used for cloning, such as *Escherichia coli* (*E. coli*), the single digit copy nature of the bacterial artificial chromosome reduces the instability, and the reduced 20 instability is thought to occur because the single copy nature limits the the ability *E. coli* to recognize cryptic promoters in the RSV cDNA and produce toxic proteins.

Respiratory Syncytial Virus (RSV)

Typically, the RSV particle contains a viral genome within a helical nucleocapsid 25 which is surrounded by matrix proteins and an envelope containing viral glycoproteins. The genome of wild-type RSV encodes the proteins, NS1, NS2, N, P, M, SH, G, F, M2-1, M2-2, and L. G, F, and SH are glycoproteins. The F gene has been incorporated into a number of viral vaccines. RSV polymerase activity consists of the large protein (L) and phosphoprotein (P). The viral M2-1 protein is used during transcription and is likely to be a 30 component of the transcriptase complex. The viral N protein is used to encapsidate the nascent RNA.

The genome is transcribed and replicated in the cytoplasm of a host cell. Host-cell transcription typically results in synthesis of ten methylated and polyadenylated mRNAs. The antigenome is positive-sense RNA complement of the genome produced during

replication, which in turn acts as a template for genome synthesis. The viral genes are flanked by conserved gene-start (GS) and gene-end (GE) sequences. At the 3' and 5' ends of the genome are leader and trailer nucleotides. The wild type leader sequence contains a promoter at the 3' end. When the viral polymerase reaches a GE signal, the polymerase 5 polyadenylates and releases the mRNA and reinitiates RNA synthesis at the next GS signal. The L-P complex is believed to be responsible for recognition of the promoter, RNA synthesis, capping and methylation of the 5' termini of the mRNAs and polyadenylation of their 3' ends. It is believed that the polymerase sometimes dissociates from the gene at the junctions. Because the polymerase initiates transcription at the 3' end of the genome, this 10 results in a gradient of expression, with the genes at the 3' end of the genome being transcribed more frequently than those at the 5' end.

To replicate the genome, the polymerase does not respond to the *cis*-acting GE and GS signals and generates positive-sense RNA complement of the genome, the antigenome. At the 3' end of the antigenome is the complement of the trailer, which contains a promoter. 15 The polymerase uses this promoter to generate genome-sense RNA. Unlike mRNA, which is released as naked RNA, the antigenome and genome RNAs are encapsidated with virus nucleoprotein (N) as they are synthesized.

In certain embodiments, the disclosure relates to vectors and nucleic acids that contain RSV gene(s) such as the wild-type genome or antigenome. An example of an RSV 20 antigenome is provided in U.S. Patent No. 6,790,449, hereby incorporated by reference. Reference to RSV gene(s) and the genome is contemplated to include certain mutations, deletions, or variant combinations, such as cold-passaged (cp) and temperature sensitive (ts) derivatives of RSV, cpRSV, such as rA2cp248/404/1030ΔSH. rA2cp248/404ΔSH contains 4 independent attenuating genetic elements: cp which is based on 5 missense mutations in 25 the N and L proteins and the F glycoprotein that together confer the non-ts attenuation phenotype of cpRSV; ts248, a missense mutation in the L protein; ts404, a nucleotide substitution in the gene-start transcription signal of the M2 gene; and ΔSH, complete deletion of the SH gene. rA2cp248/404/1030ΔSH contains 5 independent attenuating genetic elements: those present in rA2cp248/404ΔSH and ts1030, another missense 30 mutation in the L protein. See Karron et al., J Infect Dis., 2005, 191(7): 1093-1104, hereby incorporated by reference. Within certain embodiments, it is contemplated that the RSV antigenome may contain deletion or mutations in nonessential genes (e.g., the SH, NS1, NS2, and M2-2 genes) or combinations thereof.

Bacterial Artificial Chromosomes (BACs)

In certain embodiments, the disclosure relates to vectors and nucleic acids that contain bacterial artificial chromosomes. A bacterial cloning system for mapping and analysis of complex genomes has been disclosed in Shizuya et al., Proc. Natl. Acad. Sci., 5 1992, 89:8794-8797. The BAC system (for bacterial artificial chromosome) is based on Escherichia coli and its single-copy plasmid F factor which were described as useful for cloning large fragments of human DNA. The F factor encodes for genes that regulate its own replication including oriS, repE, parA, and parB. The oriS and repE genes mediate the unidirectional replication of the F factor while parA and parB typically maintain copy 10 number at a level of one or two per E. coli genome. It is contemplated that the genes and the chromosome may contain mutations, deletions, or variants with desired functional attributes. The BAC vector (pBAC) typically contains these genes as well as a resistance marker and a cloning segment containing promoters for incorporating nucleic acid segments of interest by ligating into restriction enzyme sites. Exemplary BAC systems include those 15 described in Shizuya & Kouros-Hehr, Keio J Med, 2001, 50(1): 26-30, hereby incorporated by reference.

One may reconstitute infectious RSV virus from the RSV BAC plasmids disclosed herein. BAC vectors can be transfected to bacteria such as E. coli by electroporation. The RSV-BACs disclosed herein may be stably maintained in bacteria, re-isolated from the 20 bacteria, and inserted into a eukaryotic cell along with one or more vectors that express the N, P, L, and M2-1 proteins. These cells produce infective RSV particles. Production of infectious RSV results from co-transfection of plasmids encoding N, P, L, and M2-1 proteins and the antigenome under control of the T7 promoter into BHK-21 cells that express T7 RNA polymerase (BSR cells). See Buchholz et al., J Virol., 2000, 74(3):1187- 25 1199, hereby incorporated by reference.

Vaccines

A number of attenuated RSV strains as candidate vaccines for intranasal administration have been developed using multiple rounds of chemical mutagenesis to 30 introduce multiple mutations into a virus. Evaluation in rodents, chimpanzees, adults and infants indicate that certain of these candidate vaccine strains are immunogenic, and may be attenuated. Nucleotide sequence analysis of some of these attenuated viruses indicates that each level of increased attenuation is typically associated with two or more new nucleotide and amino acid substitutions.

The disclosure provides the ability to distinguish between silent incidental mutations versus those responsible for phenotype differences by introducing the mutations, separately and in various combinations, into the genome or antigenome of infectious RSV. This process identifies mutations responsible for phenotypes such as attenuation, 5 temperature sensitivity, cold-adaptation, small plaque size, host range restriction, etc. Mutations from this menu can then be introduced in various combinations to calibrate a vaccine virus to an appropriate level of attenuation, etc., as desired. Moreover, the present disclosure provides the ability to combine mutations from different strains of virus into one strain.

10 The present disclosure also provides for methods of attenuation. For example, individual internal genes of RSV can be replaced with their bovine, murine or other RSV counterpart. This may include part or all of one or more of the NS1, NS2, N, P, M, SH, M2-1, M2-2 and L genes, or parts of the G and F genes. Reciprocally, means are provided to generate a live attenuated bovine RSV by inserting human attenuating genes into a bovine 15 RSV genome or antigenome background. Human RSV bearing bovine RSV glycoproteins provides a host range restriction favorable for human vaccine preparations. Bovine RSV sequences which can be used in the present disclosure are described in, e.g., Pastey et al., J. Gen. Virol. 76:193-197 (1993); Pastey et al., Virus Res. 29:195-202 (1993); Zamora et al., J. Gen. Virol. 73:737-741 (1992); Mallipeddi et al., J. Gen. Virol. 74:2001-2004 (1993); 20 Mallipeddi et al., J. Gen. Virol. 73:2441-2444 (1992); and Zamora et al., Virus Res. 24:115-121 (1992), each of which is incorporated herein by reference.

The disclosure also provides the ability to analyze other types of attenuating mutations and to incorporate them into infectious RSV for vaccine or other uses. For example, a tissue culture-adapted nonpathogenic strain of pneumonia virus of mice (the 25 murine counterpart of RSV) lacks a cytoplasmic tail of the G protein (Randhawa et al., Virology 207: 240-245 (1995)). By analogy, the cytoplasmic and transmembrane domains of each of the RSV glycoproteins, F, G and SH, can be deleted or modified to achieve attenuation.

Other mutations for use in infectious RSV of the present disclosure include 30 mutations in cis-acting signals identified during mutational analysis of RSV minigenomes. For example, insertional and deletional analysis of the leader and trailer and flanking sequences identified viral promoters and transcription signals and provided a series of mutations associated with varying degrees of reduction of RNA replication or transcription. Saturation mutagenesis (whereby each position in turn is modified to each of the nucleotide

alternatives) of these cis-acting signals also has identified many mutations which reduced (or in one case increased) RNA replication or transcription. Any of these mutations can be inserted into the complete antigenome or genome as described herein. Other mutations involve replacement of the 3' end of genome with its counterpart from antigenome, which is 5 associated with changes in RNA replication and transcription. In addition, the intergenic regions (Collins et al., Proc. Natl. Acad. Sci. USA 83:4594-4598 (1986), incorporated herein by reference) can be shortened or lengthened or changed in sequence content, and the naturally-occurring gene overlap (Collins et al., Proc. Natl. Acad. Sci. USA 84:5134-5138 (1987), incorporated herein by reference) can be removed or changed to a different 10 intergenic region by the methods described herein.

In another embodiment, RSV useful in a vaccine formulation can be conveniently modified to accommodate antigenic variation in circulating virus, including antigenic subgroup A and B strains and variations within those subgroups. Typically the modification will be in the G and/or F proteins. The entire G or F gene, or the segment(s) encoding 15 particular immunogenic regions thereof, is incorporated into the RSV genome or antigenome cDNA by replacement of the corresponding region in the infectious clone or by adding one or more copies of the gene such that several antigenic forms are represented. Progeny virus produced from the modified RSV cDNA are then used in vaccination protocols against the emerging strains. Further, inclusion of the G protein gene of RSV 20 subgroup B would broaden the response to cover a wider spectrum of the relatively diverse subgroup A and B strains infecting human populations.

An infectious RSV clone of the disclosure can also be engineered to enhance its immunogenicity and induce a level of protection greater than that provided by natural infection, or vice versa, to identify and ablate epitopes associated with undesirable 25 immunopathologic reactions. Enhanced immunogenicity of the vaccines produced by the present disclosure addresses one of the greatest obstacles to controlling RSV, namely the incomplete nature of immunity induced by natural infection. An additional gene may be inserted into or proximate to the RSV genome or antigenome which is under the control of an independent set of transcription signals. Genes of interest include those encoding 30 cytokines (e.g., IL-2 through IL-15, especially IL-3, IL-6 and IL-7, etc.), gamma-interferon, and proteins rich in T helper cell epitopes. The additional protein can be expressed either as a separate protein or as a chimera engineered from a second copy of one of the RSV proteins, such as SH. This provides the ability to modify and improve the immune response against RSV both quantitatively and qualitatively.

For vaccine use, virus produced according to the present disclosure can be used directly in vaccine formulations, or lyophilized, as desired, using lyophilization protocols well known to the artisan. Lyophilized virus will typically be maintained at about 4 degrees C. When ready for use the lyophilized virus is reconstituted in a stabilizing solution, e.g., 5 saline or comprising SPG, Mg, and HEPES, with or without adjuvant, as further described below.

Thus RSV vaccines of the disclosure contain as an active ingredient an immunogenetically effective amount of RSV produced as described herein. The modified virus may be introduced into a host with a physiologically acceptable carrier and/or 10 adjuvant. Useful carriers are well known in the art, and include, e.g., water, buffered water, 0.4% saline, 0.3% glycine, hyaluronic acid and the like. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration, as mentioned above. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate 15 physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents and the like, for example, sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, sorbitan monolaurate, triethanolamine oleate, and the like. Acceptable adjuvants include incomplete Freund's adjuvant, aluminum phosphate, aluminum hydroxide, or alum, which are materials well known in the art.

Upon immunization with a RSV composition as described herein, via aerosol, droplet, oral, topical or other route, the immune system of the host responds to the vaccine by producing antibodies specific for RSV virus proteins, e.g., F and G glycoproteins. As a result of the vaccination the host becomes at least partially or completely immune to RSV infection, or resistant to developing moderate or severe RSV infection, particularly of the 25 lower respiratory tract.

The host to which the vaccines are administered can be any mammal which is susceptible to infection by RSV or a closely related virus and which host is capable of generating a protective immune response to the antigens of the vaccinating strain. Thus, suitable hosts include humans, non-human primates, bovine, equine, swine, ovine, caprine, 30 lagamorph, rodents, etc. Accordingly, the disclosure provides methods for creating vaccines for a variety of human and veterinary uses.

The vaccine compositions containing the RSV of the disclosure are administered to a host susceptible to or otherwise at risk of RSV infection to enhance the host's own immune response capabilities. Such an amount is defined to be an "immunogenically

effective dose." In this use, the precise amounts again depend on the host's state of health and weight, the mode of administration, the nature of the formulation. The vaccine formulations should provide a quantity of modified RSV of the disclosure sufficient to effectively protect the host patient against serious or life-threatening RSV infection.

5 The RSV produced in accordance with the present disclosure can be combined with viruses of the other subgroup or strains to achieve protection against multiple RSV subgroups or strains, or protective epitopes of these strains can be engineered into one virus as described herein. Typically the different viruses will be in admixture and administered simultaneously, but may also be administered separately. For example, as the F 10 glycoproteins of the two RSV subgroups differ by only about 11% in amino acid sequence, this similarity is the basis for a cross-protective immune response as observed in animals immunized with RSV or F antigen and challenged with a heterologous strain. Thus, immunization with one strain may protect against different strains of the same or different subgroup.

15 In some instances it may be desirable to combine the RSV vaccines of the disclosure with vaccines which induce protective responses to other agents, particularly other childhood viruses. For example, the RSV vaccine of the present disclosure can be administered simultaneously with parainfluenza virus vaccine, such as described in Clements et al., *J. Clin. Microbiol.* 29:1175-1182 (1991), incorporated herein by reference.

20 In another aspect of the disclosure the RSV can be employed as a vector for protective antigens of other respiratory tract pathogens, such as parainfluenza, by incorporating the sequences encoding those protective antigens into the RSV genome or antigenome which is used to produce infectious RSV as described herein.

25 Single or multiple administrations of the vaccine compositions of the disclosure can be carried out. In neonates and infants, multiple, sequential administrations may be required to elicit sufficient levels of immunity. Administration may begin within the first month of life, or before, about two months of age, typically not later than six months of age, and at intervals throughout childhood, such as at two months, six months, one year and two years, as necessary to maintain sufficient levels of protection against native (wild-type) RSV 30 infection. Similarly, adults who are particularly susceptible to repeated or serious RSV infection, such as, for example, health care workers, day care workers, family members of young children, the elderly (over 55, 60, or 65 years), individuals with compromised cardiopulmonary function, may require multiple immunizations to establish and/or maintain protective immune responses. Levels of induced immunity can be monitored by measuring

amounts of neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to maintain desired levels of protection. Further, different vaccine viruses may be advantageous for different recipient groups. For example, an engineered RSV strain expressing an additional protein rich in T cell epitopes may be
5 particularly advantageous for adults rather than for infants.

In yet another aspect of the disclosure, RSV is employed as a vector for transient gene therapy of the respiratory tract. According to this embodiment, the recombinant RSV genome or antigenome incorporates a sequence which is capable of encoding a gene product of interest. The gene product of interest is under control of the same or a different
10 promoter from that which controls RSV expression. The infectious RSV produced by coexpressing the recombinant RSV genome or antigenome with the N, P, L and M2-1 proteins and containing a sequence encoding the gene product of interest is administered to a patient. Administration is typically by aerosol, nebulizer, or other topical application to the respiratory tract of the patient being treated. Recombinant RSV is administered in an
15 amount sufficient to result in the expression of therapeutic or prophylactic levels of the desired gene product. Examples of representative gene products which are administered in this method include those which encode, for example, those particularly suitable for transient expression, e.g., interleukin-2, interleukin-4, gamma-interferon, GM-CSF, G-CSF, erythropoietin, and other cytokines, glucocerebrosidase, phenylalanine hydroxylase, cystic
20 fibrosis transmembrane conductance regulator (CFTR), hypoxanthine-guanine phosphoribosyl transferase, cytotoxins, tumor suppressor genes, antisense RNAs, and vaccine antigens.

In certain embodiments, the disclosure relates to immunogenic compositions (e.g., vaccines) comprising an immunologically effective amount of a recombinant RSV of the invention (e.g., an attenuated live recombinant RSV or inactivated, non-replicating RSV), an immunologically effective amount of a polypeptide disclosed herein, and/or an immunologically effective amount of a nucleic acid disclosed herein.
25

In certain embodiments, the disclosure relates to methods for stimulating the immune system of an individual to produce a protective immune response against RSV. In
30 the methods, an immunologically effective amount of a recombinant RSV disclosed herein, an immunologically effective amount of a polypeptide disclosed herein, and/or an immunologically effective amount of a nucleic acid disclosed herein is administered to the individual in a physiologically acceptable carrier.

Typically, the carrier or excipient is a pharmaceutically acceptable carrier or excipient, such as sterile water, aqueous saline solution, aqueous buffered saline solutions, aqueous dextrose solutions, aqueous glycerol solutions, ethanol, or combinations thereof. The preparation of such solutions ensuring sterility, pH, isotonicity, and stability is effected 5 according to protocols established in the art. Generally, a carrier or excipient is selected to minimize allergic and other undesirable effects, and to suit the particular route of administration, e.g., subcutaneous, intramuscular, intranasal, oral, topical, etc. The resulting aqueous solutions can e.g., be packaged for use as is or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration

10 In certain embodiments, the RSV (or RSV components) is administered in a quantity sufficient to stimulate an immune response specific for one or more strains of RSV (e.g., an immunologically effective amount of RSV or an RSV component is administered). Preferably, administration of RSV elicits a protective immune response. Dosages and methods for eliciting a protective anti-viral immune response, adaptable to producing a 15 protective immune response against RSV, are known to those of skill in the art. See, e.g., U.S. Pat. No. 5,922,326; Wright et al. (1982) Infect. Immun. 37:397-400; Kim et al. (1973) Pediatrics 52:56-63; and Wright et al. (1976) J. Pediatr. 88:931-936. For example, virus can be provided in the range of about 10^3 - 10^6 pfu (plaque forming units) per dose administered (e.g., 10^4 - 10^5 pfu per dose administered). Typically, the dose will be adjusted based on, 20 e.g., age, physical condition, body weight, sex, diet, mode and time of administration, and other clinical factors. The prophylactic vaccine formulation can be systemically administered, e.g., by subcutaneous or intramuscular injection using a needle and syringe or a needleless injection device. Preferably, the vaccine formulation is administered intranasally, e.g., by drops, aerosol (e.g., large particle aerosol (greater than about 10 25 microns)), or spray into the upper respiratory tract. While any of the above routes of delivery results in a protective systemic immune response, intranasal administration confers the added benefit of eliciting mucosal immunity at the site of entry of the virus. For intranasal administration, attenuated live virus vaccines are often preferred, e.g., an attenuated, cold adapted and/or temperature sensitive recombinant RSV, e.g., a chimeric 30 recombinant RSV. As an alternative or in addition to attenuated live virus vaccines, killed virus vaccines, nucleic acid vaccines, and/or polypeptide subunit vaccines, for example, can be used, as suggested by Walsh et al. (1987) J. Infect. Dis. 155:1198-1204 and Murphy et al. (1990) Vaccine 8:497-502.

In certain embodiments, the attenuated recombinant RSV is as used in a vaccine and is sufficiently attenuated such that symptoms of infection, or at least symptoms of serious infection, will not occur in most individuals immunized (or otherwise infected) with the attenuated RSV - in embodiments in which viral components (e.g., the nucleic acids or 5 polypeptides herein) are used as vaccine or immunogenic components. However, virulence is typically sufficiently abrogated such that mild or severe lower respiratory tract infections do not typically occur in the vaccinated or incidental host.

While stimulation of a protective immune response with a single dose is preferred, additional dosages can be administered, by the same or different route, to achieve the 10 desired prophylactic effect. In neonates and infants, for example, multiple administrations may be required to elicit sufficient levels of immunity. Administration can continue at intervals throughout childhood, as necessary to maintain sufficient levels of protection against wild-type RSV infection. Similarly, adults who are particularly susceptible to repeated or serious RSV infection, such as, for example, health care workers, day care 15 workers, family members of young children, the elderly, and individuals with compromised cardiopulmonary function may require multiple immunizations to establish and/or maintain protective immune responses. Levels of induced immunity can be monitored, for example, by measuring amounts of virus-neutralizing secretory and serum antibodies, and dosages adjusted or vaccinations repeated as necessary to elicit and maintain desired levels of 20 protection.

Alternatively, an immune response can be stimulated by ex vivo or in vivo targeting of dendritic cells with virus. For example, proliferating dendritic cells are exposed to viruses in a sufficient amount and for a sufficient period of time to permit capture of the RSV antigens by the dendritic cells. The cells are then transferred into a subject to be 25 vaccinated by standard intravenous transplantation methods.

Optionally, the formulation for prophylactic administration of the RSV also contains one or more adjuvants for enhancing the immune response to the RSV antigens. Suitable adjuvants include, for example: complete Freund's adjuvant, incomplete Freund's adjuvant, saponin, mineral gels such as aluminum hydroxide, surface active substances such as 30 lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, bacille Calmette-Guerin (BCG), *Corynebacterium parvum*, and the synthetic adjuvant QS-21.

If desired, prophylactic vaccine administration of RSV can be performed in conjunction with administration of one or more immunostimulatory molecules. Immunostimulatory molecules include various cytokines, lymphokines and chemokines

with immunostimulatory, immunopotentiating, and pro-inflammatory activities, such as interleukins (e.g., IL-1, IL-2, IL-3, IL-4, IL-12, IL-13); growth factors (e.g., granulocyte-macrophage (GM)-colony stimulating factor (CSF)); and other immunostimulatory molecules, such as macrophage inflammatory factor, Flt3 ligand, B7.1; B7.2, etc. The 5 immunostimulatory molecules can be administered in the same formulation as the RSV, or can be administered separately. Either the protein or an expression vector encoding the protein can be administered to produce an immunostimulatory effect.

Although vaccination of an individual with an attenuated RSV of a particular strain of a particular subgroup can induce cross-protection against RSV of different strains and/or 10 subgroups, cross-protection can be enhanced, if desired, by vaccinating the individual with attenuated RSV from at least two strains, e.g., each of which represents a different subgroup. Similarly, the attenuated RSV vaccines can optionally be combined with vaccines that induce protective immune responses against other infectious agents.

15

EXPERIMENTAL

The A2-line19F-I557V virus is immunogenic in BALB/c mice

This is demonstrated in Fig. 7, which shows that this virus induces higher levels of RSV-neutralizing serum antibodies than RSV A2 and RSV A2-line19F. Figure 7B demonstrates that, even low input doses, this virus provide complete protection to challenge 20 with a heterologous strain of RSV, when challenged 29 days post-primary infection. This complete protection with low dose immunization is not seen for two other strains of RSV, A2-K-line19F and A2-K-A2GF, which allow for breakthrough reinfection. Those two viruses are similar to A2-line19F-I557V except for the F protein, indicating that the I557V F protein encoded by this virus is important for the phenotype.

25

In addition to being immunogenic (Fig. 7A), the A2-line19F-I557V virus is thermostable. Thermostability of the virus was measured as the ability of the virus to retain titer over multiple days when incubated at either 4°C or 37°C. The results indicated indicate that this virus is more thermostable than the A2-K-A2GF virus at both temperatures tested and more stable than A2-line19F at 4°C. As stated above, the F gene is the only difference 30 between these two viruses, indicating this unique F protein is responsible for the phenotype.

The A2-line 19 F RSV strain is more stable than the A2 strain, and Val at 557 in the context of the line 19 F protein makes the virus even more stable. Val at position 557 in other strains is also likely stabilizing - 557 position and stability. In certain embodiments,

the disclosure contemplates other mutations at position 557 (any amino acid, e.g., alanine, valine, isoleucine, leucine), in any F strain context, that affect thermostability of the virus.

Generation of recombinant RSV with NS1 and NS2 codon silent mutations and

5 Growth Attenuation

Codons that are uncommon in humans were used to prepare recombinant RSV with the NS1 and NS2 genes designated dNS1h and dNS2h below. Codons that are uncommon in RSV were used to prepare recombinant RSV with the NS1 and NS2 genes designated dNS1v and dNS2v below. Figure 1 provides a table used to determine optimal sequences.

10 Recombinant RSV was prepared with the following nucleotide sequences for the NS1 and NS2 gene. It is important to note that prior to testing codons, it was unpredictable if either the uncommon human codons or uncommon RSV codons would produce a desirable RSV vaccine candidate. Experiments using codons uncommon for RSV sequences had the unanticipated and undesirable effect of increased expression. Using codons uncommon for
15 human sequences had the desirable effect of decreased expression. Experiments comparing NS codons uncommon for human sequences and NS codons uncommon for RSV sequences indicated that the codons uncommon for human sequences were preferential for vaccine development.

20 dNS1h nucleotide sequence (SEQ ID NO: 6) has which as 84 out of 420 nucleotides (20%) different and 68 out of 140 codons (48%) than NS1 in wild-type A2

SEQ ID NO: 6

ATGGGTTCGAATTGCTATCGATGATAAAAGTACGTCTACAAAATCTATTGAT
25 AATGATGAAGTAGCGCTACTAAAAATAACGTGTTACGGATAAACTAATACA
TCTAACGAATGCGCTAGCGAAAGCGGTAATACATACGATAAAACTAAATGGTA
TAGTATTGTACATGTAATAACGTCGTCGGATATATGTCGAATAATAATAG
TAGTAAAATCGAATTTACGACGATGCCGGTACTACAAAATGGTGGTTATATAG
GGGAAATGATGGAACTAACGCATTGTCGCAACCGAATGGTCTACTAGATGAT
30 AATTGTGAAATAAAATTTGAAAAAACTATCGGATTGACGATGACGAATTAT
ATGAATCAACTATCGGAACTAAGGTTTGATCTAAATCCGTAA

dNS1v nucleotide sequence (SEQ ID NO: 7) has which as 145 out of 420 nucleotides (34%) different and 122 out of 140 codons (87%) than NS1 in wild-type A2

SEQ ID NO: 7

ATGGGGTCGAACTCGCTCTCGATGATCAAGGTCCGCCTCCAGAATCTCTCGAC
AACGACGAGGTCGCGCTCCTCAAGATCACGTGTTACACGGACAAGCTCATCCAC
5 CTCACGAACCGCGCTCGCGAAGGCGGTACATCCACACGATCAAGCTAACGGGAT
CGTCTTCGTCCACGTCATCACGTCGTCGGACATCTGTCCGAACAAACACATCGT
CGTCAAGTCGAACCTCACGACGATGCCGGCCTCCAGAACGGGGGTACATCTG
GGAGATGATGGAGCTCACGCACGTTCGCAGCCGAACGGGCTCCTCGACGACA
ACTGTGAGATCAAGTTCTCGAAGAAGCTCTGGACTCGACGATGACGAACACTAC
10 ATGAACCAGCTCTGGAGCTCCTCGGGTTCGACCTCAACCCGTAA

dNS2h nucleotide sequence (SEQ ID NO: 9) has which as 82 out of 420 nucleotides (21%) different and 73 out of 140 codons (58%) than NS1 in wild-type A2

15 SEQ ID NO: 9

ATGGATACGACGCATAATGATAATACGCCGAAACGTCTAATGATAACGGATAT
GCGTCCGCTATCGCTAGAAACGATAATAACGTCGCTAACGCGTGATATAAAC
GCATAAATTATATCTAATAAAATCATGAATGTATAGTACGTAAACTAGATGA
ACGTCAAGCGACGTTACGTTCTAGTAAATTATGAAATGAAACTACTACATAA
20 AGTAGGTTCGACGAAATATAAAAAATACGGAATATAATACGAAATATGGTA
CGTTCCGATGCCGATATTATAAAATCATGATGGTTCTAGAATGTATAGGTAT
AAAACCGACGAAACATACGCCGATAATATAAAATATGATCTAAATCCGTAA

25 dNS2v nucleotide sequence (SEQ ID NO: 10) has which as 103 out of 420 nucleotides (27%) different and 92 out of 140 codons (73%) than NS1 in wild-type A2

SEQ ID NO: 10

ATGGACACGACGCACAACGACAACACGCCGAGCGCCTCATGATCACGGACAT
GCGCCCGCTCTCGCTCGAGACGATCATCACGTCGCTACGCGCGACATCATCAC
30 GCACAAGTTCATCTACCTCATCAACCACGAGTGTATCGTCCGCAAGCTCGACGA
GCGCCAGGCGACGTTCACGTTCTCGTCAACTACGAGATGAAGCTCCTCCACAA
GGTCGGGTCGACGAAGTACAAGAAGTACACGGAGTACAACACGAAGTACGGG
ACGTTCCCGATGCCGATCTTCATCAACCACGACGGGTTCTCGAGTGTATCGGG

ATCAAGCCGACGAAGCACACGCCGATCATCTACAAGTACGACCTAACCCGTA
A

BEAS-2B cell lines at 60-70% confluence are infected with the recombinant virus
5 indicated as above at MOI (multiplicity of infection) of 0.01 (i.e., for each 100 cells, there is
one infectious virus particle). This is done by first counting the cells before infection,
calculating the total number of cells in each well, then calculating the amount of each virus
for infection. Infection is done at room temperature for 1 hour, then washed off. The
infected cells are left in 37° C incubator with 5% CO₂ for up to 96 hours. Samples are taken
10 at 12, 24, 48, 72, and 96 hours after infection and frozen. After collecting all the time point
samples, the amount of virus in each sample is determined by titering on Vero cell lines
according to standard protocol and the titer (FFU/mL, meaning Fluorescent Focus-forming
Unit per mL) is calculated for each sample. Since viruses used have a red fluorescent gene
in the genome, the infected cells are counted under the fluorescent microscope providing
15 fluorescent focus-forming units. Each data point represents duplicate samples from two
independent experiments.

As illustrated in Figure 2, growth of kRSV-dNS1h (human deoptimized NS1+NS2
virus) is attenuated in the BEAS-2B cell line at 72 and 96 hours post infection. It is
believed that this is due to lower NS1 and NS2 proteins than wild type virus.

20

Expression of RSV in plasmid designed for low copy number

Infectious recombinant RSV (rRSV) can be recovered from transfected plasmids.
Co-expression of RSV N, P, L, and M2 1 proteins as well as the full-length antigenomic
RNA is sufficient for RSV replication. Infectious RSV may be produced from the co-
25 transfection of plasmids encoding N, P, L, and M2-1 proteins and the antigenomic cDNA
under control of the T7 promoter into BHK-21 cells that stably express T7 RNA polymerase
(BSR cells). Currently research labs typically use a RSV antigenomic cDNA cloned in the
plasmid pBR322 (mid-range copy number, 15-20 copies per E coli). In order to maintain
the antigenomic cDNA in this plasmid, the bacteria is grown at 30° C and low aeration.
30 Nevertheless, plasmid rearrangements and clone loss is frequently experienced.

A fraction of RSV cDNA containing the attachment glycoprotein (G) and fusion (F)
genes of the virus was found to be unclonable in pUC-based plasmids (500-700 plasmid
copies in E coli). This fragment was cloned in a low copy number (approximately 5 copies

per *E. coli*) plasmid called pLG338-30.5. The plasmid pLG338-30 was developed to increase the stability of cloned lentivirus glycoproteins. Cunningham et al., Gene, 1993, 124, 93-98. It is hypothesized that cDNA instability in *E. coli* results from the presence of cryptic *E. coli* transcription promoters within viral glycoprotein sequences. Thus, instability 5 of cDNA in “promoterless” plasmids in bacteria can arise because aberrant proteins are expressed from cryptic promoters, leading to toxicity exacerbated by plasmid copy number.

An antigenomic plasmid was generated containing the RSV strain A2 genome with the strain line 19 F gene in place of the A2 F gene. It had been derived from the antigenome plasmid first disclosed in Collins et al., Proc Natl Acad Sci U S A., 1995, 10 92(25):11563-11567 and U.S. Patent No. 6,790,449 hereby incorporated by reference. The antigenome was digested out of the plasmid vector and ligated into the pKBS3 BAC.

GalK recombineering reagents were obtained from the NCI and successfully established a BAC-RSV reverse genetics protocol (Figs 4 and 5). See <http://web.ncifcrf.gov/research/brb/recombineeringInformation.aspx>, hereby incorporated 15 by reference. Mutation of RSV cDNA via BAC recombineering has enhanced the ability to manipulate RSV for generation of mutants. An added benefit of the system is enhanced stability of the full-length antigenomic cDNA in the BAC vector.

The BAC-based RSV antigenome vector was propagated at 32° C and 250 RPM without observing any vector rearrangements or clone loss in *E. coli*. Thus, BAC-RSV not 20 only enables manipulations via recombineering but also facilitates RSV reverse genetics in general owing to elimination of cDNA instability.

RSV antigenome in BAC vector (pSynkRSV_line 19 F Construction)

The RSV-BAC pSynkRSV_line 19 F contains the modified katushka gene (mKate2, 25 fluorescent protein), and restriction sites for convenient standard cloning methods. To build pSynkRSV, three nucleic acid pieces were synthesized by Gene Art, a company that synthesizes DNA. These three pieces then have to be put together in the bacterial artificial chromosome (BAC). The three pieces are designated pSynkRSV-BstBI_SacI (#1), pSynkRSV-SacI_ClaI (#2), and pSynkRSV-ClaI_MluI (#3). One uses the plasmid pKBS3 30 as the backbone for constructing pSynkRSV. See Figures 6A-E. pSynkRSV contains the bacterial artificial chromosome sequences needed to regulate copy number and partitioning in the bacteria.

To insert the three synthesized segments, one puts oligonucleotide adapters into pKBS3 between two existing restriction enzyme cut sites, BstBI and MluI.

The overhangs were designed such that the adapter would ligate into pKBS3 at the BstBI and MluI sites. Underlined sequences indicate restriction sites: SacI, ClaI, and AvrII from right to left respectively. This produces a multi-cloning site containing the restriction sites BstBI, SacI, ClaI, AvrII, and MluI, in that order, and a plasmid termed pKBS5. See 5 Figure 6A. One cuts and ligates the SacI_ClaI segment (#2) from Gene Art into pKBS5. See Figure 6B. The next one cuts and ligates the #3 segment using the enzymes AvrII and MluI (cannot use ClaI again due to an inactive ClaI restriction site in pSynkRSV- 10 ClaI_MluI). See Figure 6C. At this point, the plasmid pKBS5 contains the Gene Art sequences from SacI to ClaI, some intervening nucleotides (less than 10), and the Gene Art sequences from AvrII to MluI. One cuts and ligates the #1 segment using BstBI and SacI. See Figure 6D. This RSV BAC contains about 10 unwanted nucleotides between two ClaI sites (that from segment #2 and segment #3). Recombineering is used to delete those 15 nucleotides, thus generating pSynkRSV_line 19 F. See Figure 6E. The three segments should be ligated in this order to avoid potential interference from multiple restriction sites.

Recombinant respiratory syncytial virus (RSV) as live-attenuated vaccine (LAV)

Four expression plasmids were generated, one that expresses RSV nucleoprotein 20 (N), one that expresses RSV phosphoprotein (P), one that expresses RSV matrix 2 ORF 1 protein (M2-1), and one that expresses RSV large polymerase (L) - pA2- Nopt, pA2-Popt, pA2-M2-1opt, and pA2-Lopt. The nomenclature reflects the fact that these genes are of the A2 strain of RSV and that these cDNAs are optimized for human codon bias in order to increase expression levels in mammalian cells. Recovery of recombinant 25 RSV from cDNA includes five components: full length RNA (e.g. provided by pSynk-RSV119F), and RSV N, P, M2-1, and L proteins. The four helpers plasmids pA2-Nopt, pA2-Popt, pA2-M2-1opt, and pA2-Lopt useful for driving RSV rescue.

A recombinant respiratory syncytial virus strain A2-line19F was generated with a point mutation at residue F557, at which the isoleucine was changed to a valine (virus 30 name: A2-line19F-I557V). A protein expression plasmid was also generated which encodes the line 19 F protein with the same isoleucine to valine mutation at position 557 (protein name- line 19F-I557V). A2-line19F-I557V has higher thermostability, at 4° C and 37° C, than the A2-line 19F parent virus. This increased stability likely contributes to an increased

induction of neutralizing antibodies and protection by A2-line19F-I557V relative to A2-line 19F.

5 Development of a live-attenuated RSV vaccine has been hindered by low RSV immunogenicity in young infants, which constitute the target population, and limited genomic stability. A desirable vaccine is immunogenic and genetically and thermally stable and safe for vaccination in young infants.

RSV nonstructural (NS) proteins 1 and 2 (NS1 and NS2) are associated with inhibition of host cell interferon pathways and thus potentially limiting the immunogenicity of the virus. The small hydrophobic (SH) glycoprotein forms cationic pores in membranes, 10 modulates the host apoptotic pathways and inhibits tumor necrosis factor-a (TNF-a) signaling. SH, NS1 and NS2 are dispensable for virus replication. However, deletion of NS1 and NS2 together results in an over-attenuation. Deletion of the SH protein has little apparent effect on attenuation in experimental vaccine candidates currently being evaluated. However, deletion of SH enhances RSV replication in vitro and presumably enhances 15 expression of downstream genes, such as the antigenic G and F genes.

RSV vaccine candidates disclosed herein combine multiple technologies to overcome the challenges of poor immunogenicity and limited genetic and thermal stability in a safe viral vaccine candidate. RSV LAV OE1 combines limited expression of immune inhibitory proteins NS1 and NS2 through codon-deoptimization and SH protein through 20 deletion without the potential for rapid reversion in a stable and immunogenic viral background.

Vaccine candidates were generated using BAC-based RSV reverse genetics codon-deoptimization of nonstructural (NS) genes NS1 and NS2 were combined with the A2-line 19F gene containing a mutation at residue 557 , as well as deletion of the RSV small 25 hydrophobic (SH) glycoprotein.

OE1 Virus Genome (SEQ ID NO: 1)

RSV vaccine candidate genotype:

A2-mKate2-dNSh-deltaSH-A2G-line19F-I557V (tagged)
and A2-dNSh-deltaSH-A2G-line19F-I557V (untagged)

30 RSV attachment glycoprotein (G) is a heavily glycosylated protein , which exists in two variant forms: membrane-bound and secreted . Studies evaluating the functional role of RSV G have shown that it plays a role in inhibition of toll-like receptor activation and its secreted form likely acts as an immune antigen decoy. In addition to RSV F, G protein is also immunogenic, however due in part to its extensive glycosylation, it is a poor antigen

for generation of neutralizing antibodies. RSV G is indispensable for virus replication, but deletion results in over-attenuation . Thus, G can be considered a non-essential virulence gene.

5 An RSV A2 G protein sequence was substituted which contains a M48I mutation and has 50% of the codons deoptimized [dGm(50%)] into the background of the RSV LAV OE1 virus genome. The OE2 virus background includes codon-deoptimization of nonstructural (NS) genes NS1 and NS2 with the A2-line 19F gene containing a mutation at amino acid residue 557, as well as deletion of the RSV small hydrophobic (SH) glycoprotein.

10 OE2 Virus Genome (SEQ ID NO: 2)

RSV vaccine candidate genotype:

A2-mKate2-dNSh-deltaSH-dGm(50%)-line19F-I557V (tagged)
and A2-dNSh-deltaSH-dGm(50%)-line19F-I557V (untagged)

15 RSV LAV OE2 combines reduced expression of immune inhibitory glycoprotein G through codon-deoptimization of 50% of codons, 100% codondeoptimization of immunomodulatory proteins NS1 and NS2, and deletion of SH protein without the potential for rapid reversion in a stable and immunogenic viral background.

20 In a third vaccine candidate, an RSV A2 G protein sequence substituted with one which contains a M48I mutation and has 75% of the codons deoptimized [dGm(75%)] into the background of the RSV LAV OE1 virus genome. The OE3 virus background includes codon-deoptimization of nonstructural (NS) genes NS1 and NS2 with the A2-line 19F gene containing a mutation at residue 557, as well as deletion of the RSV small hydrophobic (SH) glycoprotein.

OE3 Virus Genome (SEQ ID NO: 3)

25 RSV vaccine candidate genotype:

A2-mKate2-dNSh-deltaSH-dGm(75%)-line19F-I557V (tagged)
and A2-dNSh-deltaSH-dGm(75%)-line19F-I557V (untagged)

30 RSV LAV OE3 combines reduced expression of immune inhibitory glycoprotein G through codon-deoptimization of 75% of codons, 100% codon deoptimization of immunomodulatory proteins NS1 and NS2, and deletion of SH protein without the potential for rapid reversion in a stable and immunogenic viral background.

An RSV A2 G protein sequence which contains a M48I mutation and has 100% of the codons deoptimized [dGm(100%)] into the background of the RSV LAV OE1 virus genome was generated. The OE4 virus background includes codon-deoptimization of

nonstructural (NS) genes NS1 and NS2 with the A2-line 19F gene containing a mutation at residue 557, as well as deletion of the RSV small hydrophobic (SH) glycoprotein.

OE4 Virus Genome (SEQ ID NO: 4)

RSV vaccine candidate genotype:

5 A2-mKate2-dNSh-deltaSH-dGm(100%)-line19F-I557V (tagged)
and A2-dNSh-deltaSH-dGm(100%)-line19F-I557V (untagged)

CLAIMS

1. An isolated recombinant nucleic acid encoding nonstructural protein 1 (NS1) and optionally further comprising NS2 of a wild-type human respiratory syncytial virus RSV, wherein the nucleotide sequence encoding NS1 comprises SEQ ID NO:6 or SEQ ID NO:7 or a variant thereof having 95% or greater sequence identity thereto.
2. The isolated recombinant nucleic acid of Claim 1 comprising SEQ ID NO: 6 or SEQ ID NO: 7.
3. A recombinant vector comprising a nucleic acid of Claim 1 or Claim 2.
4. An attenuated recombinant RSV comprising a nucleic acid of Claim 1 or Claim 2.
5. An expression system comprising a vector of Claim 3 or an attenuated recombinant RSV of Claim 4.
6. A vaccine comprising an attenuated recombinant RSV of Claim 4.
7. Use of the vaccine of Claim 6 in the manufacture of a medicament for the treatment of a subject who is younger than 6 months of age, under 1 year of age, born prematurely, has congenital heart or lung disease, is having chemotherapy or has had a transplantation, or has been diagnosed with asthma, congestive heart failure or chronic obstructive pulmonary disease, leukemia, elderly, or HIV/AIDS.
8. The use of Claim 7, wherein the medicament is combined with motavizumab, palivizumab, or another humanized monoclonal antibody directed against an epitope in the A antigenic site of the F protein of RSV.
9. An isolated recombinant nucleic acid comprising an RSV genome OE1 of SEQ ID NO: 1, OE2 of SEQ ID NO: 2, OE3 of SEQ ID NO: 3, OE4 of SEQ ID NO: 4 or variant with greater than 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% or more sequence identity thereto, wherein the genome comprises a nucleic acid of Claim 1.
10. A recombinant vector comprising a nucleic acid of Claim 9.

11. A cell comprising the recombinant vector of Claim 10.
12. The isolated recombinant nucleic acid of Claim 1, wherein the variant comprises one or more codon-deoptimized mutations in SEQ ID NO: 6 or SEQ ID NO: 7, wherein SEQ ID NO: 6 or SEQ ID NO: 7 are substituted such that a codon to produce Gly is GGT, a codon to produce Asp is GAT, a codon to produce Glu is GAA, a codon to produce His is CAT, a codon to produce Ile is ATA, a codon to produce Lys is AAA, a codon to produce Leu is CTA, a codon to produce Asn is AAT, a codon to produce Gln is CAA, a codon to produce Val is GTA, or a codon to produce Tyr is TAT, or combinations thereof.
13. A medicament comprising a combination with the vaccine of Claim 6 and motavizumab, palivizumab, or another humanized monoclonal antibody directed against an epitope in the A antigenic site of the F protein of RSV.
14. A method of treatment of a subject who is younger than 6 months of age, under 1 year of age, born prematurely, has congenital heart or lung disease, is having chemotherapy or has had a transplantation, or has been diagnosed with asthma, congestive heart failure or chronic obstructive pulmonary disease, leukemia, elderly, or HIV/AIDS comprising administering to the subject a therapeutically effective amount of the vaccine of Claim 6.
15. The method of Claim 14, wherein the vaccine is combined with motavizumab, palivizumab, or another humanized monoclonal antibody directed against an epitope in the A antigenic site of the F protein of RSV.

AA	Codon	Human	Brain	A2	2-20	Line19	3-2	3-4	12-21
		Genes							
	CCT								
	CCC								
	CCA								
	CCG								
	CAA								
	CAG								
	CCT								
	CGC								
	CGA								
	CGG								
	AGA								
	AGG								
	TCT								
	TCC								
	TCA								
	TGA								
	AGT								
	AGC								
	ACT								
	ACC								
	ACA								
	ACG								
	GTT								
	GTC								
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	TAC								

		Human	A2	2-20	Line19	3-2	3-4	12-21
AA	Codon	Brain	Genes					
	GCT							
	GCC							
Ala	GCA							
	GCG							
	TGT							
Cys	TGC							
	GAT							
Asp	GAC							
	GAA							
	GAG							
	TTT							
Phe	TTC							
	GCT							
	GCC							
	GCA							
	GGA							
	GGG							
	CAT							
His	CAC							
	ATT							
	ATC							
	ATA							
	AAA							
Lys	AAG							
	TTA							
	TTC							
	CTT							
	CTC							
	CTA							
	CTG							
	Leu							
	AAT							
	AAC							

FIG. 1

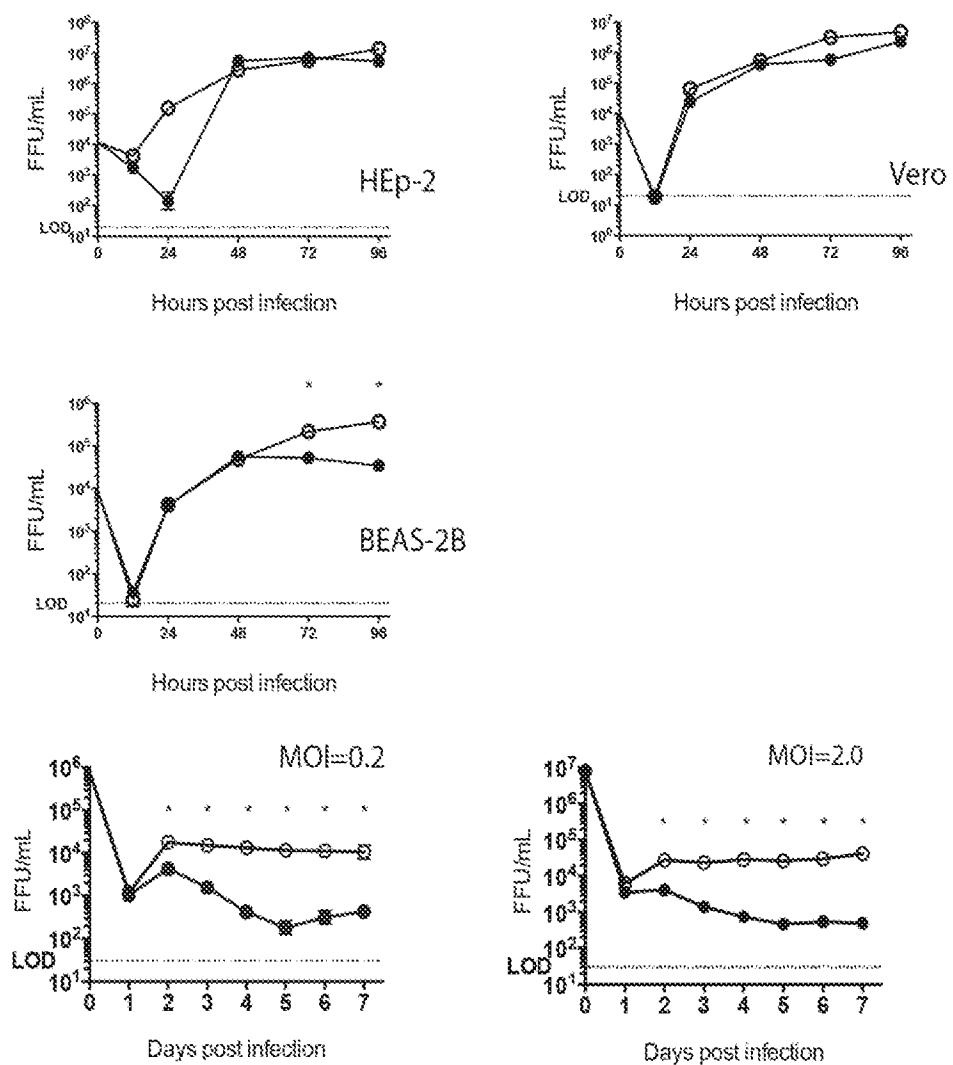
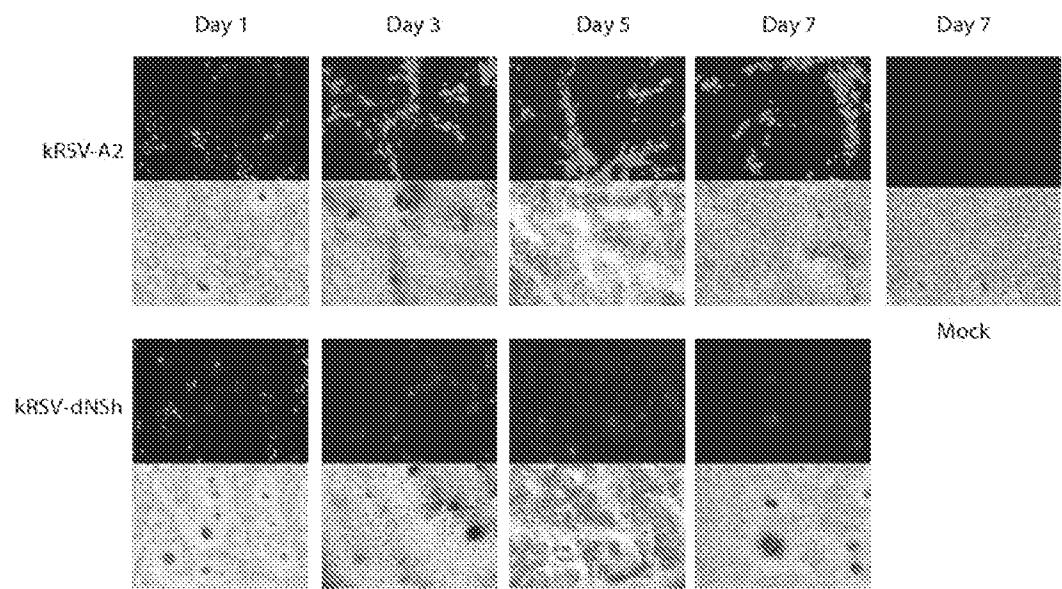


FIG. 2

**FIG. 3**

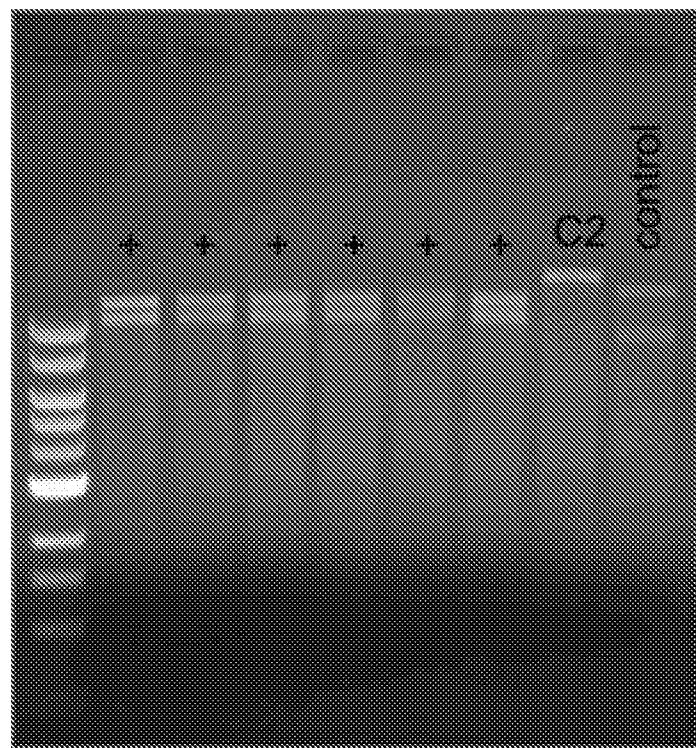


FIG. 4

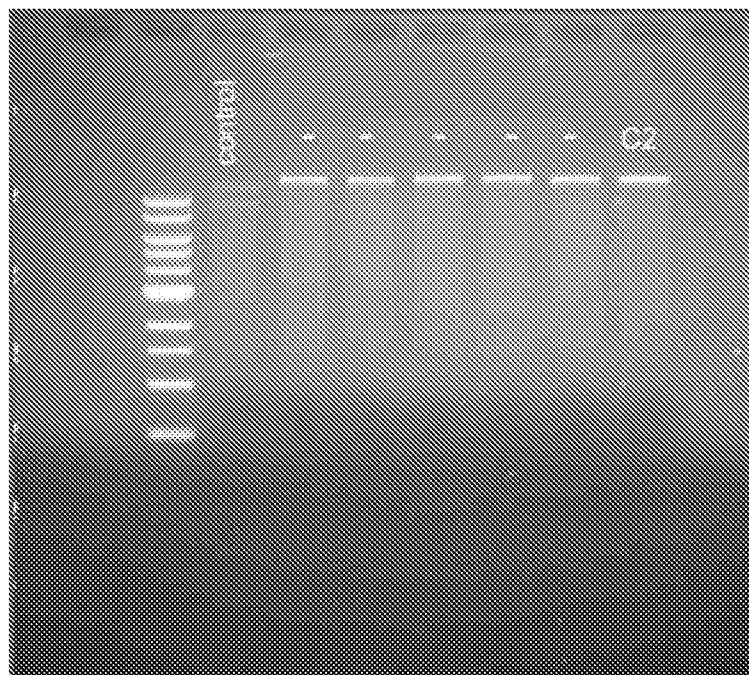


FIG. 5

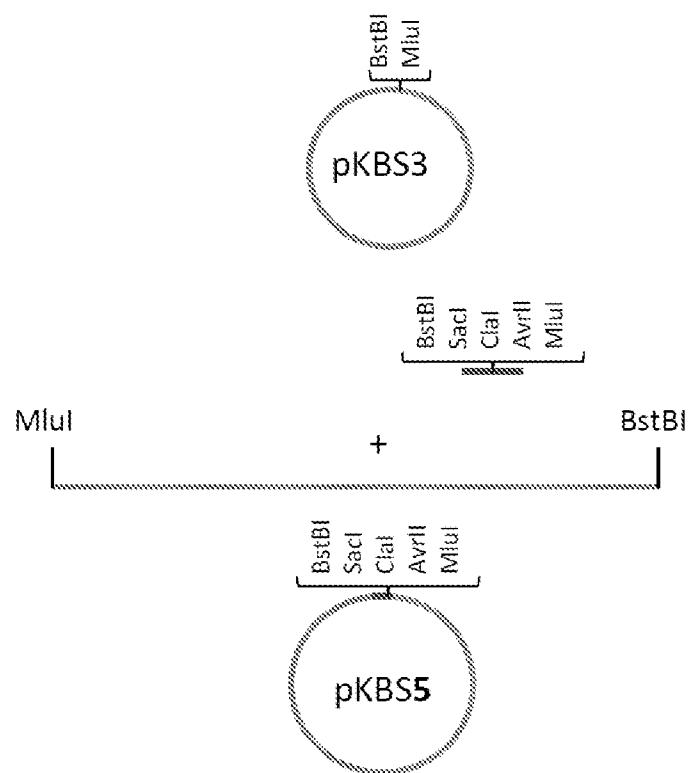


FIG. 6A

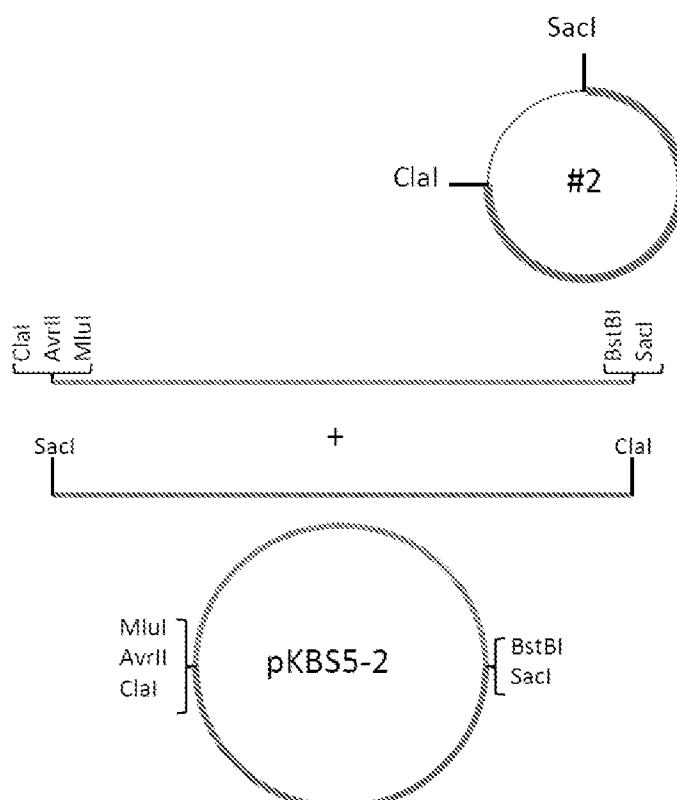


FIG. 6B

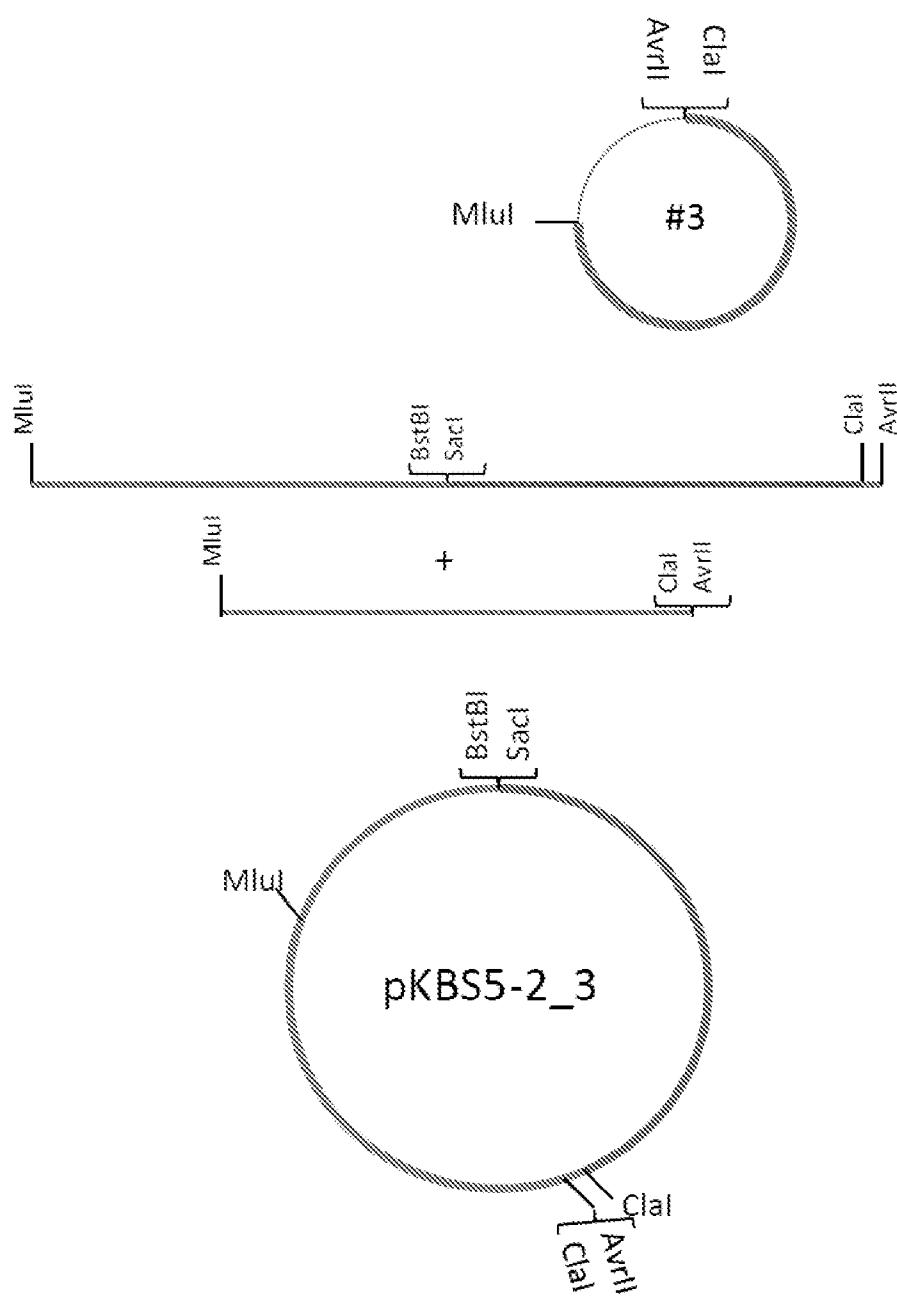


FIG. 6C

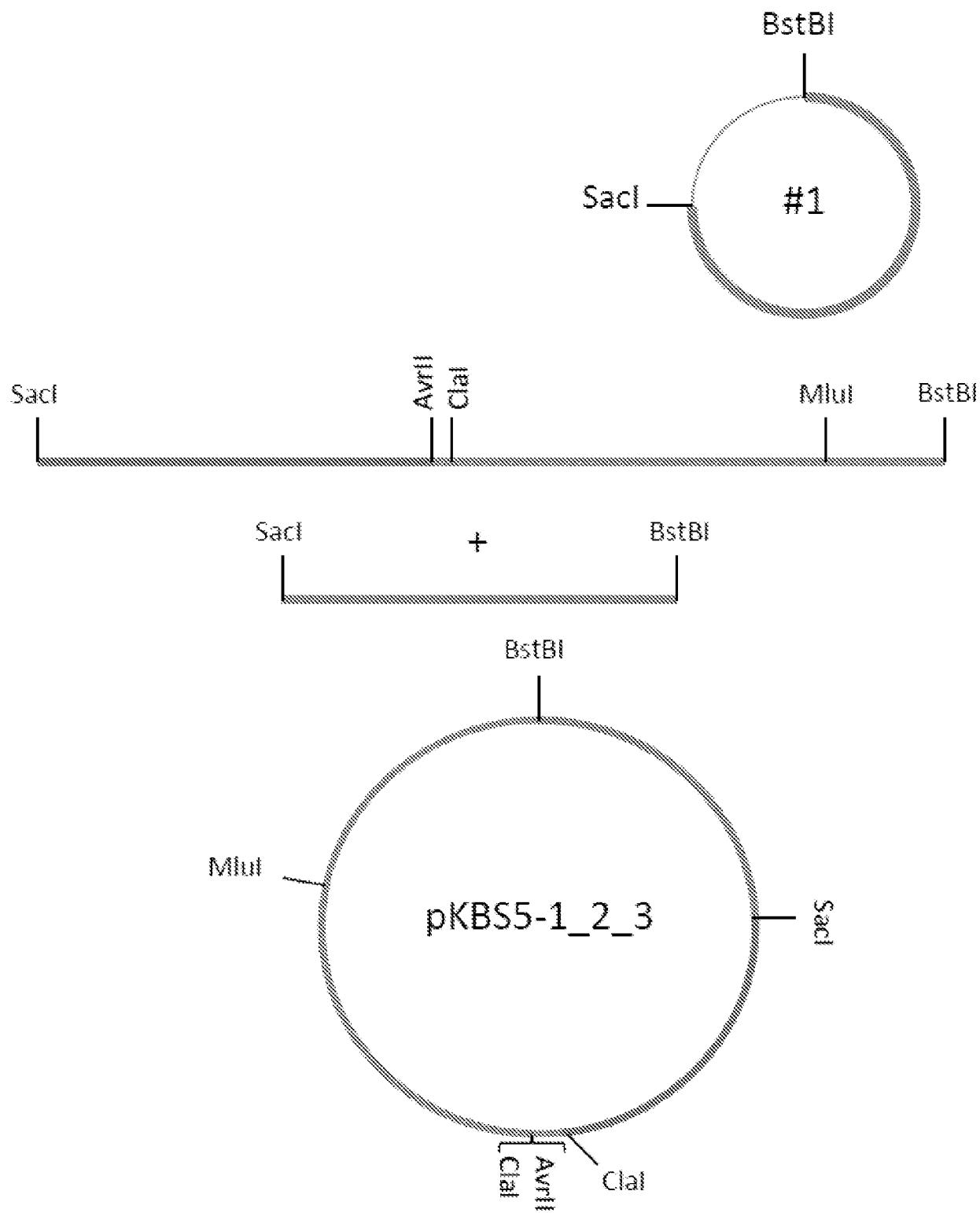


FIG. 6D

8/16

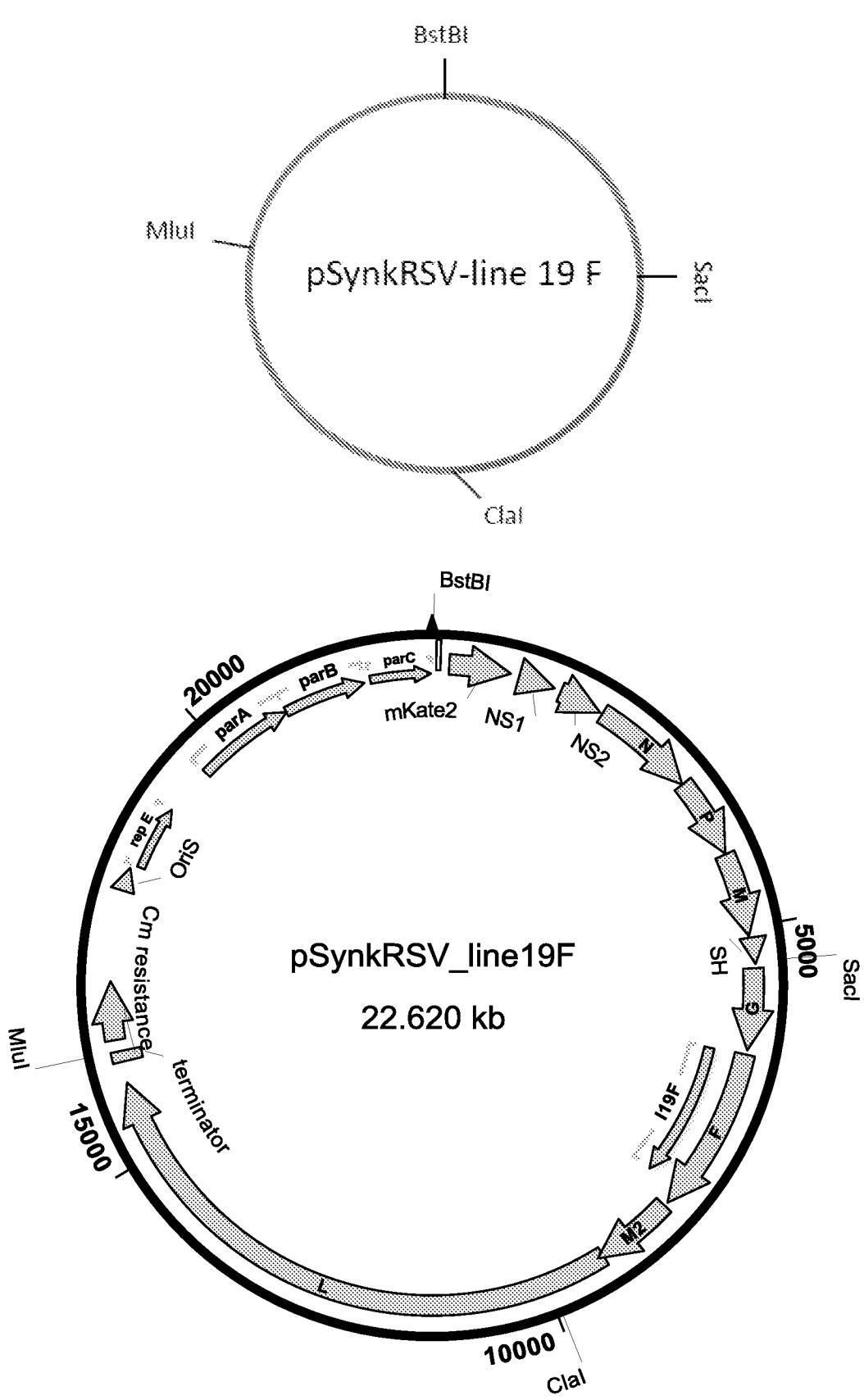


FIG. 6E

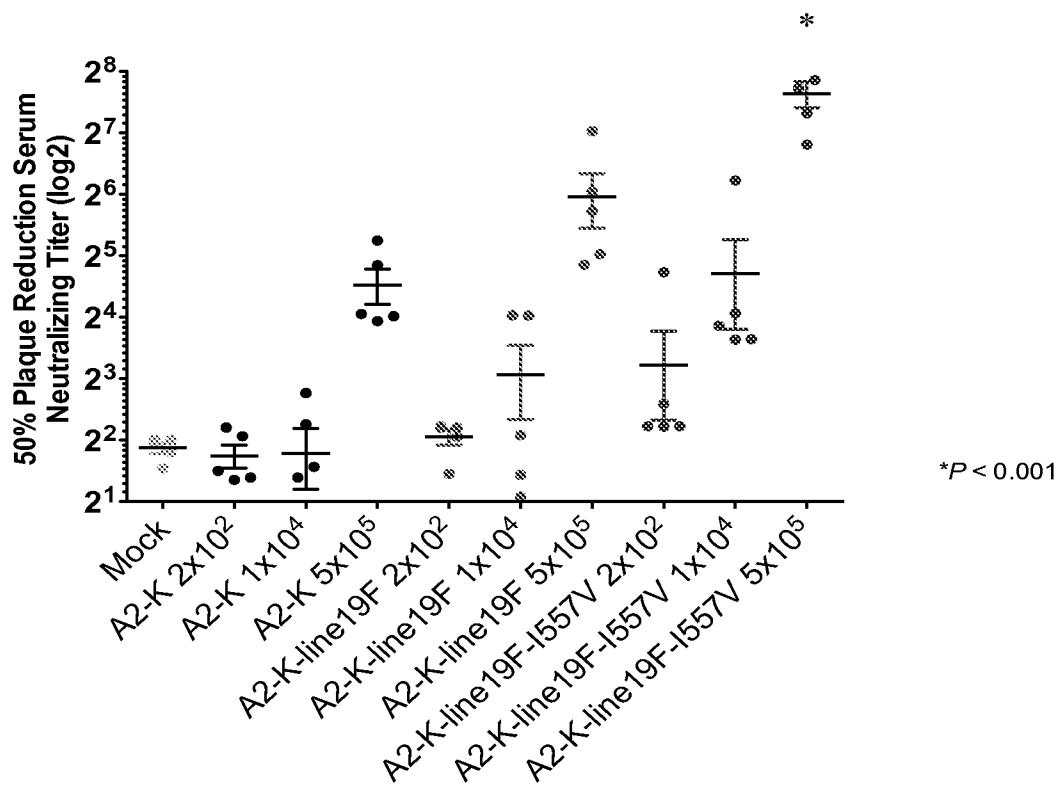


FIG. 7A

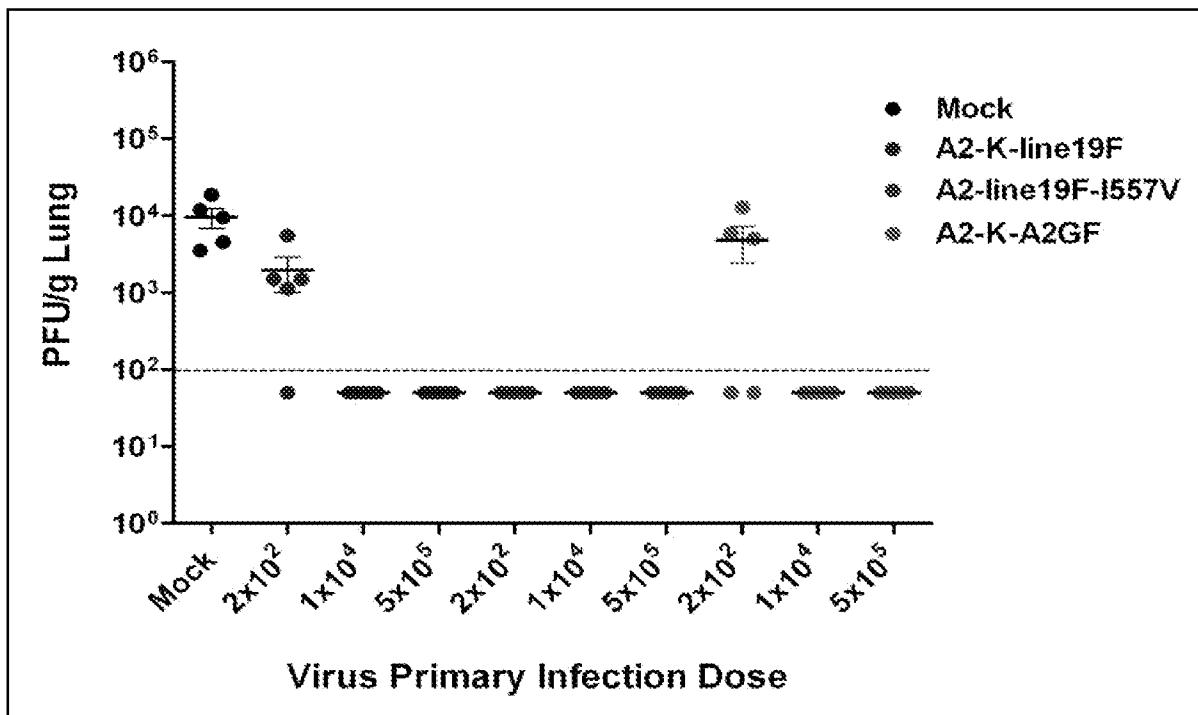


FIG. 7B

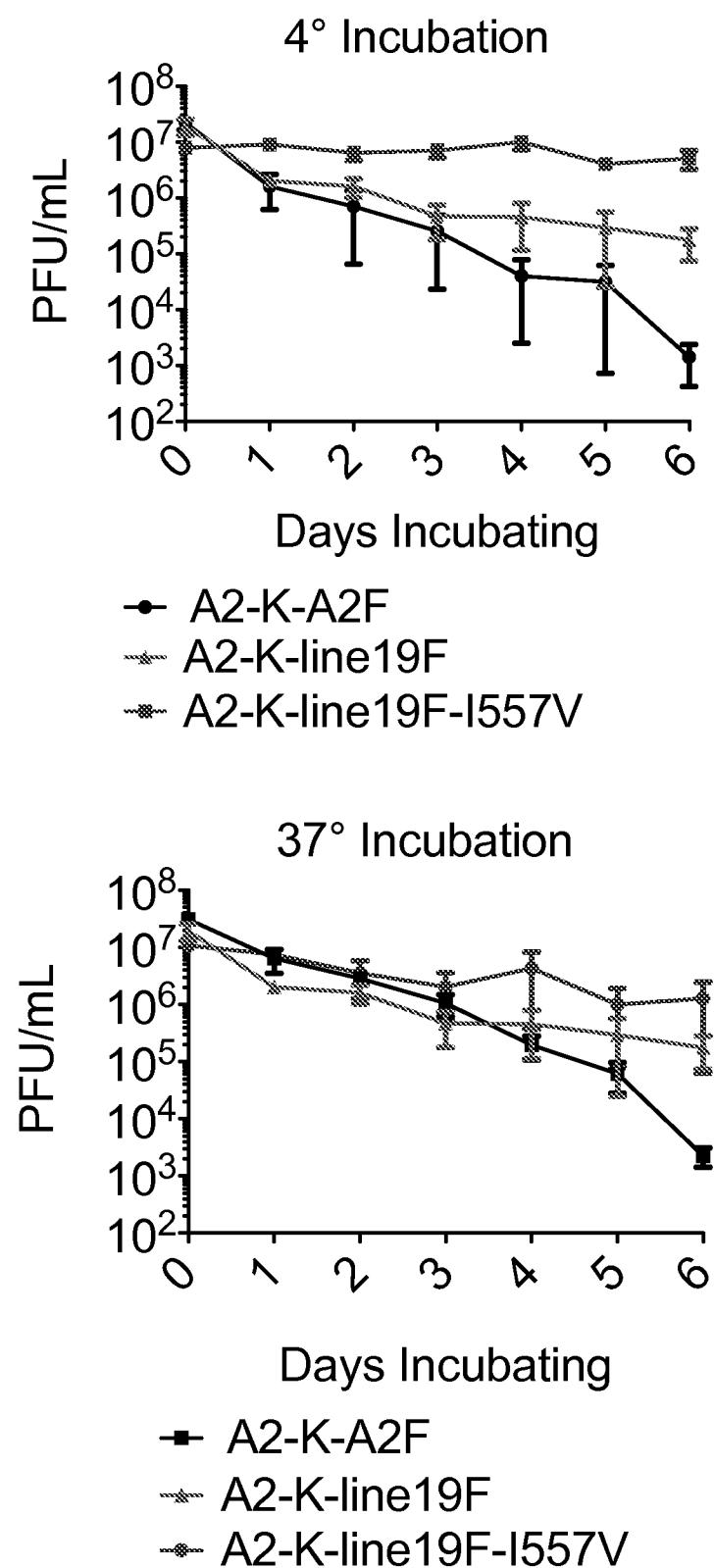


FIG. 8

Score	Expect	Method	Identities	Positives	Gaps
1169 bits(3023)	0.0	Compositional matrix adjust.	573/574(99%)	574/574(100%)	0/574(0%)
Query 1	60	MELPILKANAITTILAATVFCFASSQNITEEFYQSTCSAVSKGYL SALRTGWYTSVITIE			
60		MELPILKANAITTILAATVFCFASSQNITEEFYQSTCSAVSKGYL SALRTGWYTSVITIE			
Sbjct 1	60	MELPILKANAITTILAATVFCFASSQNITEEFYQSTCSAVSKGYL SALRTGWYTSVITIE			
120					
Query 61	120	LSNIKKNCNGTDAVKLMLQELDKYKNAVTELQLLMQSTPAANNRARRELPRFMNYTLN			
120		LSNIKKNCNGTDAVKLMLQELDKYKNAVTELQLLMQSTPAANNRARRELPRFMNYTLN			
Sbjct 61	120	LSNIKKNCNGTDAVKLMLQELDKYKNAVTELQLLMQSTPAANNRARRELPRFMNYTLN			
180					
Query 121	180	NTKKTNVTLSKKRKRRFLGFLLGVSIAASGIAVSKVLHLEGEVN KIKSALLSTNKAVVS			
180		NTKKTNVTLSKKRKRRFLGFLLGVSIAASGIAVSKVLHLEGEVN KIKSALLSTNKAVVS			
Sbjct 121	180	NTKKTNVTLSKKRKRRFLGFLLGVSIAASGIAVSKVLHLEGEVN KIKSALLSTNKAVVS			
240					
Query 181	240	LSNGVSVLTSRVLDLKNYIDKQLLPIVNQSCRISNIETVIEFQQKNNRLLEITREFSVN			
240		LSNGVSVLTSRVLDLKNYIDKQLLPIVNQSCRISNIETVIEFQQKNNRLLEITREFSVN			
Sbjct 181	240	LSNGVSVLTSRVLDLKNYIDKQLLPIVNQSCRISNIETVIEFQQKNNRLLEITREFSVN			
300					
Query 241	300	AGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNQIVRQQSYSIMSIIKEEV LAYV			
300		AGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNQIVRQQSYSIMSIIKEEV LAYV			
Sbjct 241	300	AGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNQIVRQQSYSIMSIIKEEV LAYV			
360					
Query 301	360	VQLPLYGVIDTPCWKLHTSPLCTNTKEGSNICLRTDRGWYCDNAGSVSFFPQAEKCKV			
360		VQLPLYGVIDTPCWKLHTSPLCTNTKEGSNICLRTDRGWYCDNAGSVSFFPQAEKCKV			
Sbjct 301	360	VQLPLYGVIDTPCWKLHTSPLCTNTKEGSNICLRTDRGWYCDNAGSVSFFPQAEKCKV			
420					
Query 361	420	QSNRVFCDTMYSLTLPEVNLNCVDIFNPKYDCKIMTSKTDVSSSVITSLGAI VSCY GKT			
420		QSNRVFCDTMYSLTLPEVNLNCVDIFNPKYDCKIMTSKTDVSSSVITSLGAI VSCY GKT			
Sbjct 361	420	QSNRVFCDTMYSLTLPEVNLNCVDIFNPKYDCKIMTSKTDVSSSVITSLGAI VSCY GKT			
480					
Query 421	480	KCTASNKNRGIIKTFSNGCDYVSNKGVD TVSVGNTLYYVN KQEGKSLYVKGEPI INFYDP			
480		KCTASNKNRGIIKTFSNGCDYVSNKGVD TVSVGNTLYYVN KQEGKSLYVKGEPI INFYDP			
Sbjct 421	480	KCTASNKNRGIIKTFSNGCDYVSNKGVD TVSVGNTLYYVN KQEGKSLYVKGEPI INFYDP			
540					
Query 481	540	LVFPSDEF DASISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNIMITTIIIVIIVILS			
540		LVFPSDEF DASISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNIMITTIIIVIIVILS			
Sbjct 481	540	LVFPSDEF DASISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNIMITTIIIVIIVILS			
540					
Query 541	541	LIAVGLLLYCKARSTPVTLSKDQLSGINNIAFSN 574			
541		LIAVGLLLYCKARSTP+TLSKDQLSGINNIAFSN			
Sbjct 541	541	LIAVGLLLYCKARSTPITLSKDQLSGINNIAFSN 574			

FIG. 9

Score	Expect	Method	Identities	Positives	Gaps
1160 bits(3001)	0.0	Compositional matrix adjust.	570/574(99%)	572/574(99%)	0/574(0%)
Query 60	1	MELPILKANAITTILAATVFCFASSQNITEEFYQSTCSAVSKGYL SALRTGWYTSVITIE			
Subjct 60	1	MELPILKANAITTILAATVFCFASSQNITEEFYQSTCSAVSKGYL SALRTGWYTSVITIE			
Query 120	61	LSNIKKNCNGTDAVKLMLQELDKYKNAVTELQLLMOSTPAANNRARRELPRFMNYTLN			
Subjct 120	61	LSNIK+NKCNGTDAVKLMLQELDKYKNAVTELQLLMOSTPAANNRARRELPRFMNYTLN			
Query 180	121	NTKKTNVTLSKKRKRRFLGFLLGVSIAASGIAVSKVLHLEGEVN KIKSALLSTNKAVVS			
Subjct 180	121	NTKKTNVTLSKKRKRRFLGFLLGVSIAASGIAVSKVLHLEGEVN KIKSALLSTNKAVVS			
Query 240	181	LSNGVSVLTSRVLDLKNYIDKQLLPIVNQSCRISNIETVIEFQQKNNRLLEITREFSVN			
Subjct 240	181	LSNGVSVLTS+VLDLKNYIDKQLLPIVNQSCRISNIETVIEFQQKNNRLLEITREFSVN			
Query 300	241	AGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNQIVRQQSYSIMSIIKEEV LAYV			
Subjct 300	241	AGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNQIVRQQSYSIMSIIKEEV LAYV			
Query 360	301	VQLPLYGVIDTPCWKLHTSPLCTNTKEGSNICLRTDRGWYCDNAGSVSFFPQA EKCKV			
Subjct 360	301	VQLPLYGVIDTPCWKLHTSPLCTNTKEGSNICLRTDRGWYCDNAGSVSFFPQA E CKV			
Query 420	361	QSNRVFCDTMYSLTLPEVNLCNDIFNPKYDCKIMTSKTDVSSSVITS LGAI VSCY GKT			
Subjct 420	361	QSNRVFCDTMYSLTLPEVNLCNDIFNPKYDCKIMTSKTDVSSSVITS LGAI VSCY GKT			
Query 480	421	KCTASNKRGIIKTFSNGCDYVSNKGVD TVSVGNTLYYVN KQEGKSLYVKGEPI INFYDP			
Subjct 480	421	KCTASNKRGIIKTFSNGCDYVSNKGVD TVSVGNTLYYVN KQEGKSLYVKGEPI INFYDP			
Query 540	481	LVFPSDEF DASISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNIMITTIIIVIIVILS			
Subjct 540	481	LVFPSDEF DASISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNIMITTIIIVIIVILS			
Query 541	541	LIAVGLLLYCKARSTPVLSKDQLSGINNIAFSN 574			
Subjct 541	541	LIAVGLLLYCKARSTPVLSKDQLSGINNIAFSN 574			

FIG. 10

Score	Expect	Method	Identities	Positives	Gaps
1160 bits(3002)	0.0	Compositional matrix adjust.	569/574(99%)	0/574(0%)	
Query 1 60		MELPILKANAITTILAATVFCFASSQNITEEFYQSTCSAVSKGYL SALRTGWT SVITIE MELPILKANAITTILAATVFCFASSQNITEEFYQSTCSAVSKGYL SALRTGWT SVITIE			
Sbjct 1 60		MELPILKANAITTILAATVFCFASSQNITEEFYQSTCSAVSKGYL SALRTGWT SVITIE MELPILKANAITTILAATVFCFASSQNITEEFYQSTCSAVSKGYL SALRTGWT SVITIE			
Query 61 120		LSNIKKNCNGTDAKVKLMQELDKYKNAVTELQLLMQSTPAANNRARRELPRFMNYTLN LSNIK+NKCNGTDAKVKLMQELDKYKNAVTELQLLMQSTPAANNRARRELPRFMNYTLN			
Sbjct 61 120		LSNIKENCNGTDAKVKLMQELDKYKNAVTELQLLMQSTPAANNRARRELPRFMNYTLN LSNIKENCNGTDAKVKLMQELDKYKNAVTELQLLMQSTPAANNRARRELPRFMNYTLN			
Query 121 180		NTKKTNVTLSKKRKRRFLGFLLGVSIAASGIAVSKVLHLEGEVNIKKSALLSTNKAVVS NTKKTNVTLSKKRKRRFLGFLLGVSIAASGIAVSKVLHLEGEVNIKKSALLSTNKAVVS			
Sbjct 121 180		NTKKTNVTLSKKRKRRFLGFLLGVSIAASGIAVSKVLHLEGEVNIKKSALLSTNKAVVS NTKKTNVTLSKKRKRRFLGFLLGVSIAASGIAVSKVLHLEGEVNIKKSALLSTNKAVVS			
Query 181 240		LSNGVSVLTSRVLDLK NYIDKQLLPIVNQSCRISNIETVIEFQQKNNRLLEITREFSVN LSNGVSVLTS+VLDLK NYIDKQLLPIVNQSCRISNIETVIEFQQKNNRLLEITREFSVN			
Sbjct 181 240		LSNGVSVLTSKVLDLK NYIDKQLLPIVNQSCRISNIETVIEFQQKNNRLLEITREFSVN LSNGVSVLTSKVLDLK NYIDKQLLPIVNQSCRISNIETVIEFQQKNNRLLEITREFSVN			
Query 241 300		AGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNQIVRQQSYSIMSIIKEEVLAYV GVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNQIVRQQSYSIMSIIKEEVLAYV			
Sbjct 241 300		VGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNQIVRQQSYSIMSIIKEEVLAYV VGVTPVSTYMLTNSELLSLINDMPITNDQKKLMSNNQIVRQQSYSIMSIIKEEVLAYV			
Query 301 360		VQLPLYGVIDTPCWKLHTSPLCTNTKEGSNICLRTDRGWYCDNAGSVSFFPQAECCKV VQLPLYGVIDTPCWKLHTSPLCTNTKEGSNICLRTDRGWYCDNAGSVSFFPQAECCKV			
Sbjct 301 360		VQLPLYGVIDTPCWKLHTSPLCTNTKEGSNICLRTDRGWYCDNAGSVSFFPQAETCKV VQLPLYGVIDTPCWKLHTSPLCTNTKEGSNICLRTDRGWYCDNAGSVSFFPQAETCKV			
Query 361 420		QSNRVFCDTMYSLTLPEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAI VSCY GKT QSNRVFCDTMYSLTLPEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAI VSCY GKT			
Sbjct 361 420		QSNRVFCDTMNSLTLPEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAI VSCY GKT QSNRVFCDTMNSLTLPEVNLCNVDIFNPKYDCKIMTSKTDVSSSVITSLGAI VSCY GKT			
Query 421 480		KCTASNKRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNQEGKSLYVKGEPI INFYDP KCTASNKRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNQEGKSLYVKGEPI INFYDP			
Sbjct 421 480		KCTASNKRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNQEGKSLYVKGEPI INFYDP KCTASNKRGIIKTFSNGCDYVSNKGVDTVSVGNTLYYVNQEGKSLYVKGEPI INFYDP			
Query 481 540		LVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNIMITTIIIVIIVILLS LVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNIMITTIIIVIIVILLS			
Sbjct 481 540		LVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNIMITTIIIVIIVILLS LVFPSDEFDASISQVNEKINQSLAFIRKSDELLHNVNAGKSTTNIMITTIIIVIIVILLS			
Query 541		LIAVGLLLYCKARSTPVTLSKDQLSGINNIAFSN 574			
Sbjct 541		LIAVGLLLYCKARSTPVTLSKDQLSGINNIAFSN 574			

FIG. 11

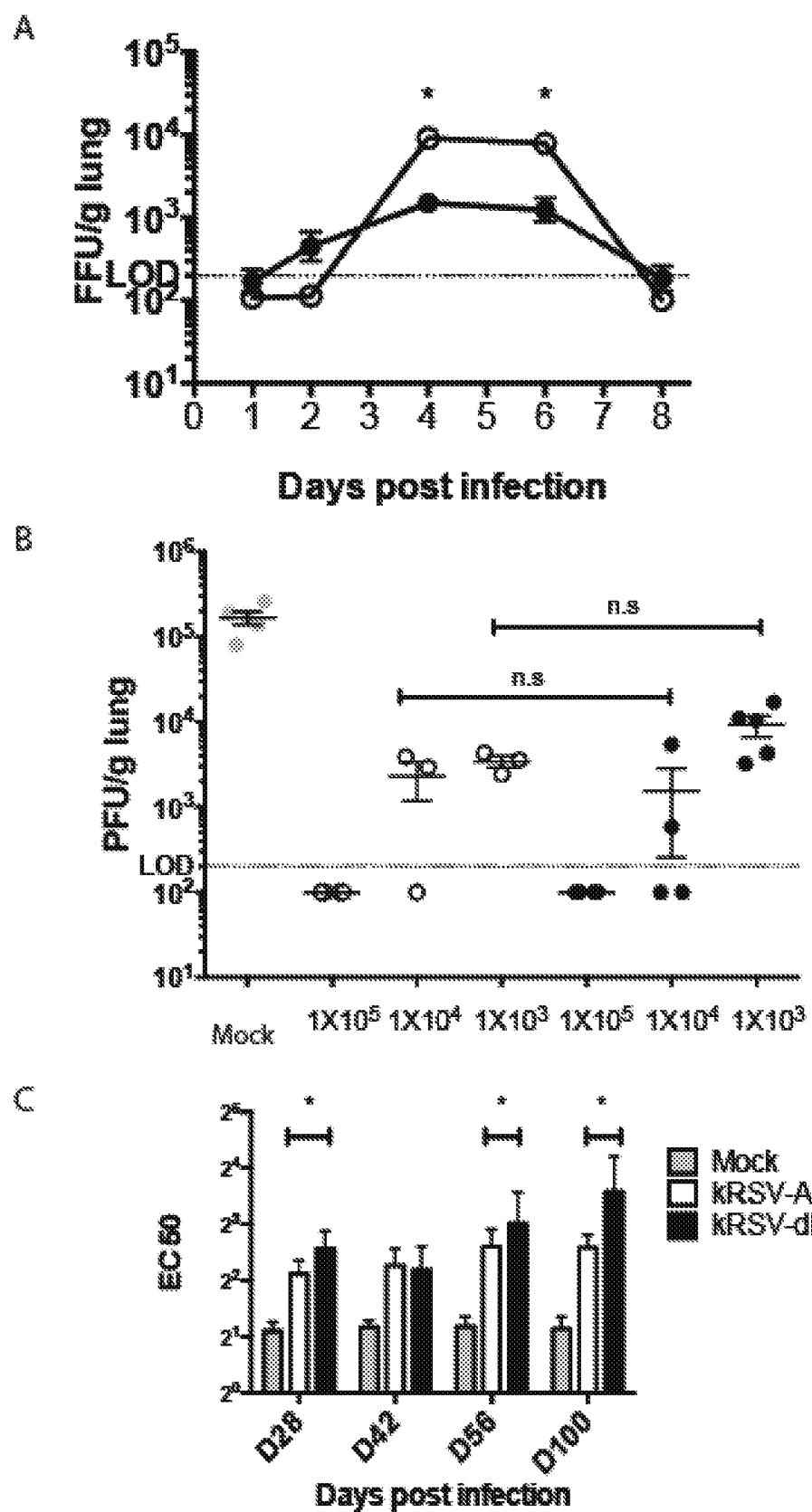
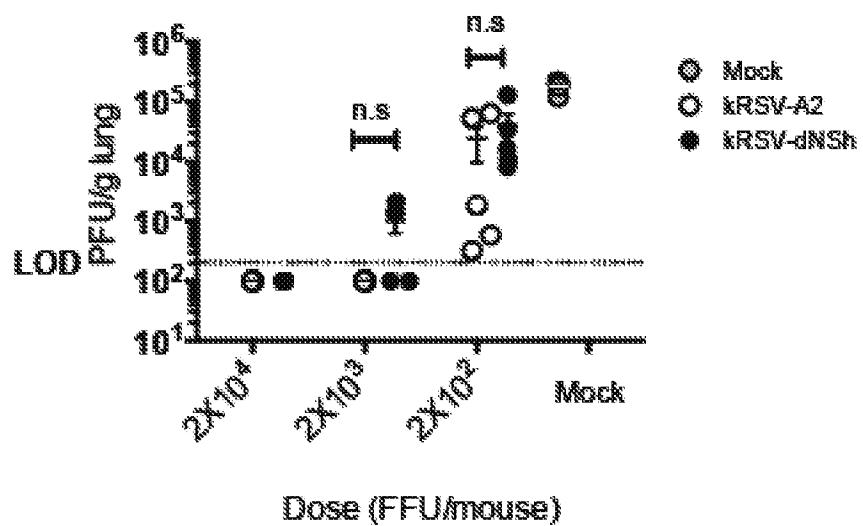


FIG. 12

A



B

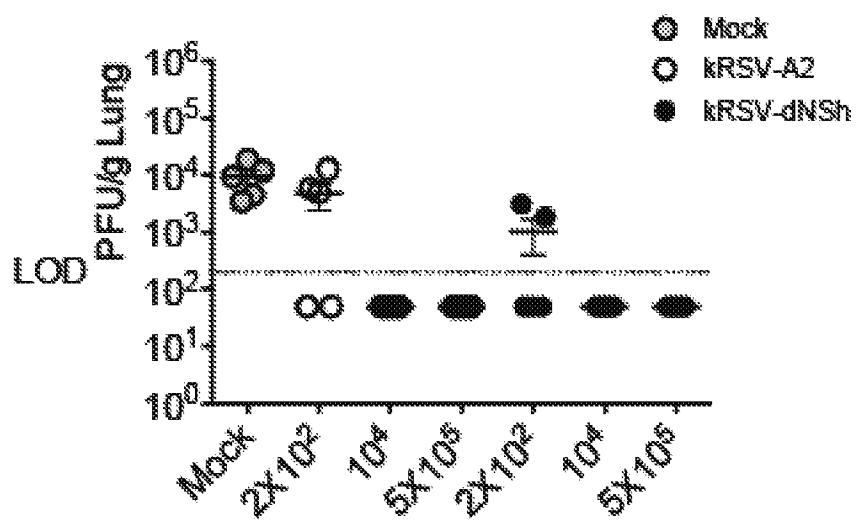


FIG. 13

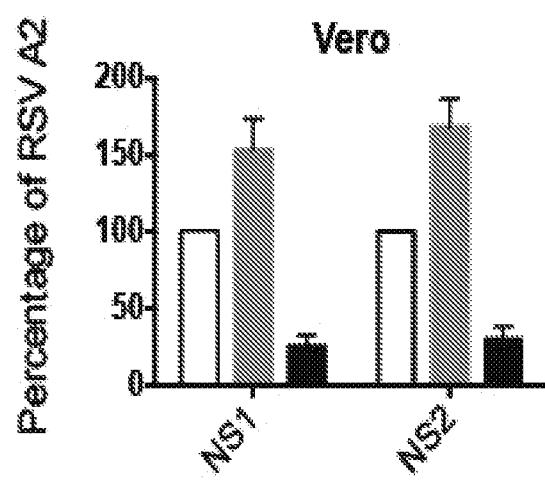
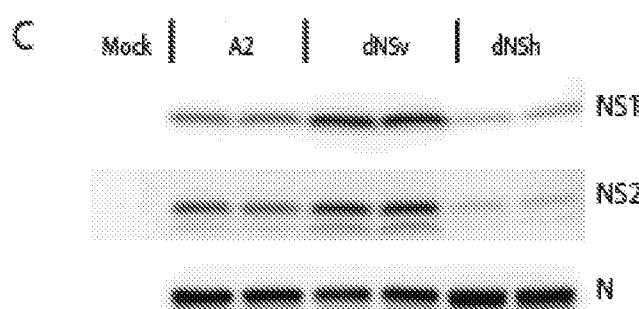
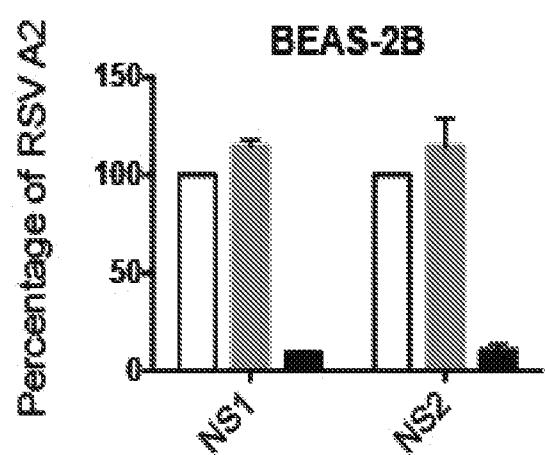
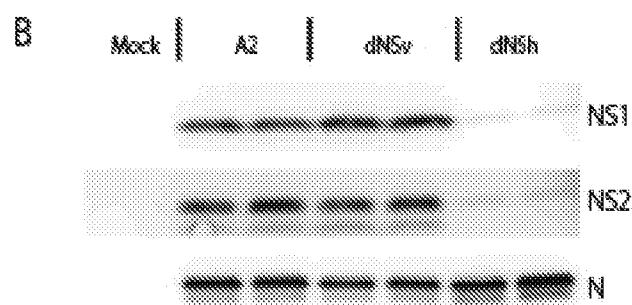
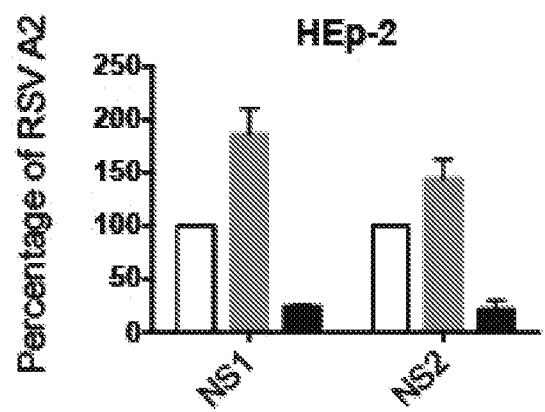
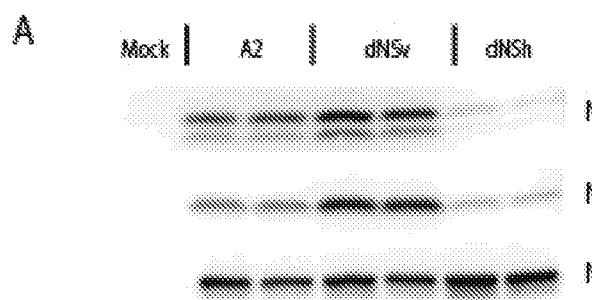


FIG. 14